

BY

PETER J. SIMS

FOR

COMPOSITIONS AND METHODS TO INHIBIT

FORMATION OF THE C5b-9 COMPLEX OF COMPLEMENT

FOR

UNITED STATES LETTERS PATENT

BY

PETER J. SIMS

FOR

COMPOSITIONS AND METHODS TO INHIBIT

FORMATION OF THE C5b-9 COMPLEX OF COMPLEMENT

**COMPOSITIONS AND METHODS TO INHIBIT
FORMATION OF THE C5b-9 COMPLEX OF COMPLEMENT**

5

The present invention is generally in the area of compounds regulating complement-mediated inflammation, and is specifically directed to compounds interacting with assembly of the C5b-9 complex.

10 The U.S. government has certain rights in this invention by virtue of grant HL36061 from the Heart, Lung and Blood Institute, National Institutes of Health to Peter J. Sims.

15 The complement system is a complex interaction of plasma proteins and membrane cofactors which act in a multi-step, multi-protein cascade sequence in conjunction with other immunological systems of the body to provide immunity from intrusion of foreign cells. Complement proteins represent up to about 10% of globulins in the normal serum of man and other vertebrates.

20 The classic complement pathway involves an initial antibody recognition of, and binding to, an antigenic site (SA) on a target cell. This surface bound antibody subsequently reacts with the first component of complement, C1q, forming a C1-antibody complex with Ca^{+2} , C1r, and C1s which is proteolytically active. C1s cleaves C2 and C4 into active components, C2a and C4a. The C4b,2a complex is an active protease called C3 convertase, and acts to cleave C3 into C3a and C3b. C3b forms a
25 complex with C4b,2a to produce C4b,2a,3b, which cleaves C5 into C5a and C5b. C5b combines with C6. The C5b,6 complex combines with C7 to form the ternary complex C5b,6,7. The C5b,6,7 complex binds C8 at the surface of the cell, which may develop functional membrane lesions and undergo slow lysis. Upon binding of C9 to the C8 molecules in the
30 C5b,6,7,8 complex, lysis of bacteria and other foreign cells is rapidly accelerated.

5 The C5b-9 proteins of the human plasma complement system have
been implicated in non-lytic stimulatory responses from certain human
vascular and blood cells. The capacity of C5b-9 to modify membrane
permeability and to selectively alter ion conductance is thought to elicit these
non-lytic responses from human cells. In the case of human blood platelets
and vascular endothelium, assembly of the C5b-9 complex initiates a transient
and reversible depolarization of the plasma membrane potential, a rise in
cytosolic Ca^{+2} , metabolic conversion of arachidonate to thromboxane or
prostacyclin, and the activation of intracellular protein kinases. In addition,
10 human platelets exposed to C5b-9 undergo shape changes, secretory fusion of
intracellular storage granules with plasma membrane, and the vesiculation of
membrane components from the cell surface. Human endothelial cells
exposed to the human C5b-9 proteins secrete high molecular weight
multimers of the platelet adhesion protein, von Willibrand Factor (vWF), and
15 the intracellular granule membrane protein, P-selectin (GMP140), is
translocated from the Weibel-Palade body to the endothelial surface. High
molecular weight multimers of vWF have been implicated in the pathogenesis
of vaso-occlusive platelet adherence to endothelium and cell surface P-
selectin (GMP140) has been implicated in the adherence of inflammatory
20 leukocytes to endothelium.

These effects of complement proteins C5b-9 on platelet and
endothelial cells alter the normal regulation of the enzymes of the plasma
coagulation system at these cell surfaces. For example, the generation of
platelet membrane microparticles by vesiculation is accompanied by the
25 exposure of membrane binding sites for coagulation factor Va. Binding of
factor Va to the platelet plasma membrane and to these membrane
microparticle sites initiates assembly of the prothrombinase enzyme complex.
This complex in turn accelerates coagulation factor Xa activation of
prothrombin to thrombin which promotes plasma clotting. Similarly, C5b-9
30 binding to the endothelial cell results in the exposure of plasma membrane

receptors for the prothrombinase complex, thereby accelerating the generation of thrombin from prothrombin at the endothelial surface.

This interaction between components of the complement and coagulation systems at the surface of blood platelets and endothelium can generate inflammatory and chemotactic peptides at sites of vascular thrombus formation and may contribute to the altered hemostasis associated with immune disease states. In addition, immune reactions affecting blood platelets and endothelium can lead to platelet aggregation, the secretion of proteolytic enzymes and vasoactive amines from platelet storage granules, and increase adherence of platelets and leukocytes to the endothelial lining of blood vessels.

Assembly of the C5b-9 complex is normally limited in plasma by the amount of C5b generated by proteolysis of C5 to its biologically-active fragments C5b and C5a. In addition to plasmin and other plasma or cell-derived proteases, two enzymes of the complement system can cleave C5 to C5a and C5b, the membrane-stabilized enzyme complexes C4b2a and C3bBb (C5-convertases). The activity of these two enzymes is normally inhibited on the surface of human blood and vascular membranes by the plasma membrane proteins, "membrane cofactor protein" (CD46), described by Lublin and Atkinson, Current Topics Microbiol. Immunol. 153:123 (1989) and "decay-accelerating factor: (CD55), Medof, et al., J. Exp. Med. 160:1558 (1984).

Platelet and endothelial cell activation by C5b-9 also has ramifications in autoimmune disorders and other disease states. The importance of spontaneous complement activation and the resulting exposure of platelets and endothelium to activated C5b-9 to the evolution of vaso-occlusive disease is underscored by consideration that a) leukocyte infiltration of the subendothelium, which is known to occur in regions of atheromatous degeneration and suggests localized generation of C5a at the vessel wall, is potentially catalyzed by adherent platelets and b) local intravascular

complement activation resulting in membrane deposition of C5b-9 complexes accompanies coronary vessel occlusion and may affect the ultimate extent of myocardial damage associated with infarction.

There is now considerable evidence that the human erythrocyte membrane as well as the plasma membranes of other human blood cells and vascular endothelium are normally protected from these effects of complement by cell-surface proteins that specifically inhibit activation of the C5b-9 pore upon C9 binding to membrane C5b-8, as reported by Holguin, M.H., et al., J. Clin. Invest. 84, 7-17 (1989); Sims, P.J., et al., J. Biol. Chem. 264, 19228-19235 (1989); Davies, A., et al., J. Exp. Med. 170, 637-654 (1989); Rollins, S.A., and Sims, P.J. J. Immunol. 144, 3478-3483 (1990); and Hamilton, K.K., et al., Blood 76, 2572-2577 (1990). Plasma membrane constituents reported to exhibit this activity include homologous restriction factor (HRF) (C8-binding protein), as described by Zalman, L.S., et al., Proc. Natl. Acad. Sci., U.S.A. 83, 6975-6979 (1986) and Schonermark, S., et al., J. Immunol. 136, 1772-1776 (1986), and the leukocyte antigen CD59, described by Sugita, Y., et al., J. Biochem. (Tokyo) 104, 633-637 (1988); Holguin, M.H., et al., (1989); Sims, P.J., et al., (1989); Davies, A., (1989); Rollins, S.A., and Sims, P.J. (1990); and Hamilton, K.K., et al., (1990).

Accumulated evidence suggest that these two proteins exhibit quite similar properties, including the following: both HRF and CD59 are tethered to the cell surface by a glycolipid anchor, and are deleted from the membranes of the most hemolytically sensitive erythrocytes that arise in the stem cell disorder paroxysmal nocturnal hemoglobinuria; the activity of both inhibitors is species-restricted, showing selectivity for C8 and C9 that are derived from homologous (i.e. human) serum; and both HRF and CD59 appear to function by inhibiting the activation of C9, decreasing the incorporation of C9 into the membrane C5b-9 complex, and limiting propagation of the C9 homopolymer. Whereas the molecular identity of

CD59 is now well-established, no peptide or cDNA sequence has yet been reported for HRF and its molecular identity remains unresolved (Sugita, Y., et al., J. Biochem. (Tokyo) 104, 633-637 (1988); Holguin, M.H., et al., (1989); Sims, P.J., et al., (1989); Davies, A. (1989); Rollins, S.A., and
5 Sims, P.J. (1990)).

Human (hu) CD59 antigen is a 18-21 kDa plasma membrane protein that functions as an inhibitor of the C5b-9 membrane attack complex (MAC) of human complement. CD59 interacts with both the C8 and C9 components of MAC during its assembly at the cell surface, thereby inhibiting formation
10 of the membrane-inserted C9 homopolymer responsible for MAC cytolytic activity. This serves to protect human blood and vascular cells from injury arising through activation of complement in plasma, as described in U.S. Patent No. 5,136,916 to Sims and Wiedmer. CD59's inhibitory activity is dependent upon the species of origin of C8 and C9, with greatest inhibitory
15 activity observed when C9 is from human or other primates. By contrast, CD59 exerts little or no inhibitory activity towards C8 or C9 of most other species, including rabbit (rb). Because the activity of CD59 is largely restricted to regulating hu C9, and the activity of analogous complement inhibitors expressed by cells of other species is likewise generally selective
20 for homologous C9, xenotypic cells and tissue are particularly susceptible to complement-mediated destruction due to unregulated activity of MAC. This phenomenon underlies hyperacute immune rejection after xenotransplantation.

Analysis of the physical association of CD59 with components of MAC suggested that separate binding sites for CD59 are contained within the
25 α -chain of hu C8 and within hu C9. The complement-inhibitory activity of CD59 is species-selective, and is most effective towards C9 derived from human or other primate plasma. The species-selective activity of CD59 was used to map the segment of human C9 that is recognized by this MAC inhibitor, using recombinant rabbit/human C9 chimeras that retain lytic
30 function within the MAC (Husler T, Lockert DH, Kaufman KM, Sodetz JM,

Sims PJ (1995) *J. Biol. Chem.* 270:3483-3486). These experiments indicated that the CD59 recognition domain was contained between residues 334-415 in human C9, as described in PCT/US96/17940 "C9 Complement Inhibitor" by Oklahoma Medical Research Foundation.

5 It is apparent that additional or alternative inhibitors of the assembly of the C5b-9 complex would be advantageous in modulation of complement-mediated inflammation. It is also clear that inhibitors which are extremely specific and which are directed to the most critical regions involved in assembly or function of the complex would be most effective as inhibitors of
10 complement-mediated inflammation, with the least likelihood of non-specific side effects.

It is therefore an object of the present invention to provide a method and materials for specifically modulating complement-mediated inflammation mediated by CD59 binding to C9.

15 **Summary of the Invention**

Compounds modulating CD59 mediated complement activity, compositions including these compounds, and methods of making and using the compounds are disclosed, which are based on the identification of the hu CD59 amino acid residues which serve as the binding site for CD59-C9
20 interactions. These residues correspond to amino acid residues 42-58, and bind to the region of C9 corresponding to human 334-418, more specifically, between amino acid residues 359 and 384. Compounds can be derived using this basic amino acid sequence and corresponding three dimensional structure within the protein using any of several techniques known to those skilled in
25 the art, including rational drug design using computer data bases and modeling of peptide/protein-ligand binding, antibodies and anti-idiotypic antibodies generated to the proteins or peptides containing this peptide sequence, and modified peptides. Those compounds imitating the structure and/or function of the peptide region are referred to herein as
30 "peptidomimetics", and include small molecules which present the surface

exposed side chains in these amino acids in the same relative positions, compounds identified by combinatorial chemistry techniques which bind to the active portions of human C9, as well as modified peptides.

The compounds can be used to inhibit complement by binding to C9 analogously to CD59, or to maintain complement inhibition, by blocking CD59 binding to C9. The compounds can be administered locally or systemically in any suitable carrier in an amount effective to either inhibit complement or block the inhibition of complement, in a patient in need of treatment thereof.

Brief Description of the Drawings

Figure 1A shows the alignment of the amino acid sequences of human (hu) and rabbit (rb) CD59 (Sequence ID No. 1 and Sequence ID No. 2, respectively). Figure 1B shows the alignment of the domains of hu and rb CD59.

Figures 2A, 2B, and 2C are schematics showing the chimeric hu/rb CD59 constructs (Figure 2A), and graphs of cytolysis (percent release of BCECF Dye versus CD59 per cell (arbitrary fluorescence units) for the human/rabbit chimeras assayed using hu C8/C9 (Figure 2B) or rb C8/C9 (Figure 2C).

Figures 3A, 3B, and 3C are schematics showing the chimeric hu/rb CD59 constructs (Figure 3A), and graphs of cytolysis (percent release of BCECF Dye versus CD59 per cell (arbitrary fluorescence units) for the human/rabbit chimeras assayed using hu C8/C9 (Figure 3B) or rb C8/C9 (Figure 3C).

Figures 4A, 4B, and 4C are schematics showing the chimeric hu/rb CD59 constructs (Figure 4A), and graphs of cytolysis (percent release of BCECF Dye versus CD59 per cell (arbitrary fluorescence units) for the human/rabbit chimeras assayed using hu C8/C9 (Figure 4B) or rb C8/C9 (Figure 4C).

Figures 5A, 5B, and 5C are schematics showing the chimeric hu/rb CD59 constructs (Figure 5A), and graphs of cytolysis (percent release of BCECF Dye versus CD59 per cell (arbitrary fluorescence units) for the human/rabbit chimeras assayed using hu C8/C9 (Figure 5B) or rb C8/C9 (Figure 5C).

Figures 6A, 6B, and 6C are schematics showing the chimeric hu/rb CD59 constructs (Figure 6A), and graphs of cytolysis (percent release of BCECF Dye versus CD59 per cell (arbitrary fluorescence units) for the human/rabbit chimeras assayed using hu C8/C9 (Figure 6B) or rb C8/C9 (Figure 6C).

Figures 7A, 7B, and 7C are schematics showing the chimeric hu/rb CD59 constructs (Figure 7A), and graphs of cytolysis (percent release of BCECF Dye versus CD59 per cell (arbitrary fluorescence units) for the human/rabbit chimeras assayed using hu C8/C9 (Figure 7B) or rb C8/C9 (Figure 7C).

Figure 8 is a sequence alignment of the amino acid sequences for CD59 of human, baboon, African green monkey, owl monkey, marmoset, HVS-15, pig, sheep, rabbit, rat, and mouse origin. Human is Sequence ID No. 3, baboon is Sequence ID No. 4, African green monkey (Afr grn mnky) is Sequence ID No. 5, owl monkey is Sequence ID No. 6, marmoset is Sequence ID No. 7, HVS-15 is Sequence ID No. 8, pig is Sequence ID No. 9, sheep is Sequence ID No. 10, rabbit is Sequence ID No. 11, rat is Sequence ID No. 12, and mouse is Sequence ID No. 13.

Figures 9A and 9B are schematics of hu/rb C9 chimeric constructs (Figure 9A) and a plot of the inhibitory activity of the chimeric C9 constructs (Figure 9B). Bar graph (right panel) summarizes combined results of all experiments measuring the inhibitory activity of CD59 with the recombinant human/rabbit chimeras of C9. In each assay, hemolytic titrations of C9 were performed against C5b-8 chE in the presence and absence of membrane CD59 and the percent reduction of hemolysis due to CD59 (*ordinate*) was

determined, with normalization to that observed for hu C9 (100% inhibition). Error bars denote mean +S.D., *parentheses* indicate number of independent experiments; *asterisks* (*) indicate significance ($p < 0.01$) when compared to rb C9; *pound signs* (#) indicate significance ($p < 0.01$) when compared to hu C9. To the left of each data bar, the protein assayed is depicted so as to designate those portions of the polypeptide containing hu C9 (*open*) or rb C9 (*shaded*) sequence. Numbers above each construct indicate the junctional hu C9 residue at each transition between human and rabbit protein sequence. Bars designated as *human C9* and *rabbit C9* denote recombinantly-expressed hu and rb C9, respectively. *Recombinant C9 chimeras* (designated #1-12) contain human (H) or rabbit (R) sequence according to the deduced mature primary structure of hu and rb C9. In some C9 chimeras, the numbering appears discontinuous because of gaps in the alignment of the human and rabbit sequences: 1, R1-338H334-415R425-536; 2, R1-363H359-538; 3, H1-357R363-536; 4, R1-363H359-415R425-536; 5, R1-363H359-391R401-536; 6, R1-400H392-415R425-536; 7, R1-363H359-384R394-536; 8, H1-333R339-424H416-538; 9, H1-357R363-424H416-538; 10, H1-357R363-400H392-538; 11, H1-391R401-424H416-538; 12, H1-357R363-393H385-538.

Figure 10 is a schematic representation of the segment of hu C9 identified as containing the CD59 binding site, which according to the proposed domain structure includes: thrombospondin type 1 (TS), LDL-receptor (LDLR), hinge (Hinge), membrane binding (MB), and epidermal growth factor precursor (EGFP) domains. *Shaded* segment indicates residues 334-415 of hu C9, spanning the putative CD59 binding site. The amino acid sequence of this peptide segment (Sequence ID No. 14) is given below, and is shown in an alignment with rb C9 (Sequence ID No. 15) (alignment done for full-length polypeptides with the PALIGN program in PCGENE). *Asterisks* indicate sequence identity. Dotted lines indicate the Cys 359/384

disulfide of hu C9 and the assumed corresponding internal disulfide in rb C9. Residue numbers refer to the mature proteins.

Figure 11 is a graph showing percent inhibitory activity of CD59 is unaffected by disruption of the Cys 359/384 disulfide. Recombinant hu C9 was expressed with Cys→Ala mutation at either residue 384 or at both residues 359/384, and analyzed as described in Figure 9B. Inhibitory activity of CD 59 measured as hemolytic function of each recombinant C9 is expressed as a percentage, relative to that measure for wild-type hu C9 (*ordinate*). Error bars denote mean +S.D., *n*, indicates number of independent experiments; asterisks indicate significance (*p*,0.001) compared to hu C9. Hu C9 and rb C9 denote the wild type human and rabbit proteins, respectively.

Figure 12 is a graph showing CD59 specifically binds hu C9 peptide 359-384. Microplates were coated with hu C9 peptide 359-384 coupled to BSA, and specific binding of biotin-CD59 determined in the presence of affinity-purified antibody against hu C9 residues 359-384 (●), or non-immune IgG (Δ) (micrograms/ml IgG concentration indicated on *abscissa*). All data were corrected for nonspecific binding of CD59, determined in presence of 20-fold excess of unlabeled CD59. *Ordinate* denotes absorbance at 405 nm, with correction for nonspecific background. Error bars denote mean +S.D. Data of a single experiment, representative of three so performed.

Figures 13A, 13B, 13C and 13D are graphs showing the inhibition of C9-dependent hemolysis by antibody against C9-peptide 359-384. Fab of antibody against hu C9 peptide 359-384 (●) was tested for its capacity to inhibit the hemolytic activity of recombinant hu C9 (Figure 13A), hu/rb C9 chimera #7 (Figure 13B), recombinant rb C9 (Figure 13C), or hu/rb C9 chimera #12 (Figure 13D). Residues of human (H) and rabbit (R) sequence in each C9 chimera are indicated in Figure 9A. Also shown is data for non-

immune antibody (Δ) (final concentrations indicated on *abscissa*). In all experiments, C5b-8 chE lacking CD59 served as target cells and hemolysis measured with correction for nonspecific lysis. Data of single experiment, representative of three similar experiments.

5

Detailed Description of the Invention

Compounds modulating CD59 mediated complement activity, compositions including these compounds, and methods of making and using the compounds are disclosed, which are based on the identification of the hu
10 CD59 amino acid residues which serve as the binding site for CD59-C9 interactions. These residues correspond to amino acid residues 42-58 (amino acids 42 to 58 of SEQ ID NO:3), and bind to the region of C9 corresponding to human 334-418, more specifically, between amino acid residues 359 and 384. Compounds can be derived using this basic amino acid sequence and
15 corresponding three dimensional structure within the protein using any of several techniques known to those skilled in the art, including rational drug design using computer data bases and modeling of peptide/protein-ligand binding, antibodies and anti-idiotypic antibodies generated to the proteins or peptides containing this peptide sequence, and modified peptides. Those
20 compounds imitating the structure and/or function of the peptide region are referred to herein as "peptidomimetics", and include small molecules which present the surface exposed side chains in these amino acids in the same relative positions, compounds identified by combinatorial chemistry techniques which bind to the active portions of human C9, as well as
25 modified peptides.

As described in PCT/US96/17940 "C9 Complement Inhibitor" by Oklahoma Medical Research Foundation, hu CD59 interacts with a segment of human C9 (hu C9) between residues 334-415, immediately C-terminal to the predicted membrane-inserting domain of C9. This segment of C9
30 contains a region of markedly divergent sequence when hu C9 is compared to

C9 of other species, with greatest divergence noted for the peptide segment contained within an internal Cys359-Cys384 disulfide in hu C9.

Human CD59 shows negligible complement inhibitory activity toward rabbit C5b-9, and rabbit CD 59 shows negligible complement inhibitory activity toward human C5b-9. Rabbit and human C5b-9 proteins work interchangeably and can assemble into functional lytic C5b-9 complexes. Recombinant DNA techniques, described in more detail in the Examples, were used to prepare various chimeric rabbit/human proteins to map the specific peptide residues involved in the binding interaction between CD59 and the C8 and C9 components of the C5b-9 complex to determine the sequence contained in CD59 which affords species specificity. It has been discovered that the entire species selective recognition of hu CD59 is encoded by amino acid residues 42-58 of human CD59. Although described herein with reference to the amino acid residues of the human proteins, it is understood that there are corresponding regions in other molecules of different origin, and that one skilled in the art would modify molecules of a different species of origin for use in that species in the same manner as described herein with reference to human CD59 and hu C9.

Unless otherwise noted, all references to amino acid positions in CD59 refer to the numbering of amino acids where the first amino acid in mature CD59 is amino acid number 1. Thus, amino acids 42-58 of CD59 refers to the forty-second through fifty-eighth amino acids of mature CD59. This numbering is used in Figures 1A and 8 and SEQ ID NO:3. SEQ ID NO:1 includes the signal peptide sequence. Rabbit CD59 contains an additional Ser residue before the highly-conserved N-terminal Leu¹ found in all other CD59 homologues that have been sequenced to date. Therefore, in order to simplify discussion of the aligned residues of hu and rb CD59 in various chimeric constructs, residues of the mature rb CD59 polypeptide have been renumbered herein commencing with [N-Ser⁰]-Leu¹-Met²-Cys³- etc.

Figure 1). All references to amino acids in rb C59 are based on this re-numbering of residues in the mature polypeptide.

The active surface exposed side chains that are available to bind C8/C9 were identified from the solution structure of hu CD59, as determined from published NMR data and the knowledge of the active portion of the CD59 molecule. These side chains are the side chains of histidine at position 44, asparagine at position 48, aspartic acid at position 49, threonine at positions 51 and 52, arginine at position 55 and glutamic acid at position 58. Compounds which present the side chains at the same relative positions will behave in a manner similar to hu CD59. NMR structures for CD59 are described in deposits by Kieffer et al., Human Complement Regulatory Protein CD59 (Extracellular Region, Residues 1 - 70) (NMR, 10 Structures), MMDB Id: 891, PDB Id: 1ERH; Kieffer et al., Human Complement Regulatory Protein CD59 (Extracellular Region, Residues 1 - 70) (NMR, 15 Restrained), MMDB Id: 890, PDB Id: 1ERG; Fletcher et al., CD59 Complexed With Glcnac-Beta-1,4-(Fuc-Alpha-1,6)-Glcna-Beta-1 (Nmr, 10 Structures), MMDB Id: 498, PDB Id: 1CDS; Fletcher et al., CD59 Complexed With Glcnac-Beta-1,4-Glcna-Beta-1 (Nmr, 10 Structures), MMDB Id: 497, PDB Id: 1CDR. The 1CDS and 1CDR deposits by Fletcher et al. are preferred.

Compounds which bind to the active portion of hu C9 can inhibit complement activity by performing substantially the same role as hu CD59. Compounds which bind to the active portion of hu CD59 can block the ability of CD59 to inhibit complement activity.

25 **Compounds Useful as Mimics of hu CD59 and C9**

A. Hu CD59 and C9 Chimeras

As described in the examples, chimeric proteins have been made which substitute the critical amino acid residues for binding activity in C9 or CD59 of one species, typically human, into a protein of a different species. The examples demonstrate how these chimeric proteins are useful to conduct

studies of function and structure, as well as to generate antibodies to the critical regions, as described below. The compounds can also be used, for example, for competitive binding assays, combinatorial chemistry to isolate compounds which only bind to the active portion of hu CD59, and as
5 therapeutics, although their therapeutic uses may be limited to non-human animals.

B. Antibodies to amino acid residues 42-58 of hu CD59 and amino acid residues 359-384 of hu C9.

Antibodies immunoreactive with the hu CD59 peptide (amino acid
10 residues 42-58), the hu C9 peptide (amino acid residues 359-384), or an anti-idiotypic antibody to these antibodies immunoreactive with the CD59 or C9 peptides can be prepared using the techniques described herein.

1. In vivo Immunization of Animals

Animals such as mice may be immunized by administration of an
15 amount of immunogen such as the CD59 peptide, an antibody to the CD59 peptide, the C9 peptide or an antibody to the C9 peptide, effective to produce an immune response. Preferably a mouse is subcutaneously injected in the back with 100 micrograms of antigen, followed three weeks later with an intraperitoneal injection of 100 micrograms of immunogen with adjuvant,
20 most preferably Freund's complete adjuvant. Additional intraperitoneal injections every two weeks with adjuvant, preferably Freund's incomplete adjuvant, may be necessary until the proper titer in the mouse's blood is achieved. In order to use the mice for fusion and hybridoma production, a titer of at least 1:5000 is preferred, and a titer of 1:100,000 or more is most
25 preferred.

In a preferred embodiment, the antibodies are raised by standard immunization of rabbits with chimeric rabbit/human CD59 protein in which human CD59 residues 42-58 replace the corresponding residues in the rabbit CD59 polypeptide or chimeric rabbit/human C9 protein in which human C9
30 residues 359-384 replace the corresponding residues in the rabbit C9

polypeptide, based upon alignment of the human and rabbit protein sequences. In another embodiment, these antibodies are raised by standard immunization of mice with chimeric mouse/human CD59 protein in which human CD59 residues 42-58 replace the corresponding residues in the mouse CD59 polypeptide, based upon alignment of the human and mouse protein sequences. The antibodies can then be purified, for example, by affinity purification on columns containing human CD59.

2. In vitro Immunization

The technique of *in vitro* immunization of human lymphocytes is frequently employed to generate a large variety of human monoclonal antibodies. Techniques for *in vitro* immunization of human lymphocytes are well known to those skilled in the art. See, e.g., T. Inai, *et al.*, Histochemistry (Germany), 99(5):335-362 (May 1993); A. Mulder, *et al.*, Hum. Immunol., 36(3):186-192 (Mar. 1993); H. Harada, *et al.*, J. Oral Pathol. Med. (Denmark), 22(4):145-152 (April 1993); N. Stauber, *et al.*, J. Immunol. Methods (Netherlands), 161(2):157-168 (May 26, 1993); and S. Venkateswaran, *et al.*, Hybridoma, 11(6) 729-739 (Dec. 1992). These techniques can be used to produce antigen-reactive human monoclonal antibodies, including antigen-specific IgG, and IgM human monoclonal antibodies.

3. Humanization of Antibodies

Since the methods for immunizing animals yield antibody which is not of human origin, the antibodies could elicit an adverse effect if administered to humans. Methods for "humanizing" antibodies, or generating less immunogenic fragments of non-human antibodies, are well known. A humanized antibody is one in which only the antigen-recognized sites, or complementarity-determining hypervariable regions (CDRs) are of non-human origin, whereas all framework regions (FR) of variable domains are

products of human genes. These "humanized" antibodies present a less xenograft rejection stimulus when introduced to a human recipient.

To accomplish humanization of a selected mouse monoclonal antibody, the CDR grafting method described by Daugherty, *et al.*, Nucl. Acids Res., 19:2471-2476 (1991) may be used. Briefly, the variable region DNA of a selected animal recombinant anti-idiotypic ScFv is sequenced by the method of Clackson, T., *et al.*, Nature, 352:624-688, 1991. Using this sequence, animal CDRs are distinguished from animal framework regions (FR) based on locations of the CDRs in known sequences of animal variable genes. Kabat, H.A., *et al.*, Sequences of Proteins of Immunological Interest, 4th Ed. (U.S. Dept. Health and Human Services, Bethesda, MD, 1987). Once the animal CDRs and FR are identified, the CDRs are grafted onto human heavy chain variable region framework by the use of synthetic oligonucleotides and polymerase chain reaction (PCR) recombination. Codons for the animal heavy chain CDRs, as well as the available human heavy chain variable region framework, are built in four (each 100 bases long) oligonucleotides. Using PCR, a grafted DNA sequence of 400 bases is formed that encodes for the recombinant animal CDR/human heavy chain FR protection.

The immunogenic stimulus presented by the monoclonal antibodies so produced may be further decreased by the use of Pharmacia's (Pharmacia LKB Biotechnology, Sweden) "Recombinant Phage Antibody System" (RPAS), which generates a single-chain Fv fragment (ScFv) which incorporates the complete antigen-binding domain of the antibody. In the RPAS, antibody variable heavy and light chain genes are separately amplified from the hybridoma mRNA and cloned into an expression vector. The heavy and light chain domains are co-expressed on the same polypeptide chain after joining with a short linker DNA which codes for a flexible peptide. This assembly generates a single-chain Fv fragment (ScFv) which incorporates the complete antigen-binding domain of the antibody. Compared to the intact

monoclonal antibody, the recombinant ScFv includes a considerably lower number of epitopes, and thereby presents a much weaker immunogenic stimulus when injected into humans.

4. Use of Chimeras to Select Particularly Active Antibodies

Antibodies which bind to inactive portions of the peptides will be less effective than antibodies which bind to the active portions. One can use the chimeric peptides described above to select particularly preferred antibodies.

For example, once an antibody is prepared, it can be bound to the appropriate peptide (hu CD59 or C9), and chimeras containing the active portion of the peptide from the rabbit sequence and the remainder of the peptide containing the human sequence can be used in a competitive binding study. If the chimera is effective at competitively binding with the antibody, the antibody is likely binding to an inactive portion of the peptide. A chimera containing the active portion of the peptide from the human sequence and the inactive portion of the peptide from the rabbit sequence can also be used in a competitive binding study. If the chimera is effective at competitively binding with the antibody, the antibody is likely bound to the active portion of the peptide.

C. Compounds Identified by Combinatorial Chemistry

Molecules with a given function, catalytic or ligand-binding, can be selected for from a complex mixture of random molecules in what has been referred to as "in vitro genetics" (Szostak, TIBS 19:89, 1992) or combinatorial chemistry. One synthesizes a large pool of molecules bearing random and defined sequences and subjects that complex mixture, for example, approximately 10^{15} individual sequences in 100 μ g of a 100 nucleotide RNA, to some selection and enrichment process. For example, by repeated cycles of affinity chromatography and PCR amplification of the molecules bound to the ligand on the column, Ellington and Szostak (1990) estimated that 1 in 10^{10} RNA molecules folded in such a way as to bind a

given ligand. DNA molecules with such ligand-binding behavior have been isolated (Ellington and Szostak, 1992; Bock et al, 1992).

Using methodology well known to those of skill in the art, in combination with various combinatorial libraries, one can isolate and
5 characterize those compounds which bind to or interact with the desired target. The relative binding affinity of these compounds can be compared and optimum compounds identified using competitive binding studies which are well known to those of skill in the art.

Identification of compounds which bind the active portion of the hu
10 CD59 and hu C9 can be simplified using the data regarding the active portions of these molecules identified using chimeric molecules. For example, the human CD59 or C9 can be bound to a solid support, and interacted with various combinatorial libraries. Those molecules which do not bind these molecules at all are removed immediately by elution with a
15 suitable solvent. Those molecules which bind to inactive portions of the CD59 or C9 molecules can be removed by competitive binding with an excess of a chimeric peptide with the inactive portions represented by human sequences and the active portion represented by the rabbit sequence. Those compounds which bind to the active portion of the CD59 or C9 will remain
20 bound to the solid support, whereas compounds bound to inactive portions of these molecules will be removed from the column. Finally, those compounds still bound to the column (which are bound to the active portions of these molecules) can be removed, for example, by competitive binding with CD59 or C9, or chimeras including only the active human portion of these
25 molecules. Following removal, these compounds can be identified and their relative binding affinity compared as described above.

D. Rational Drug Design

Drugs with the ability to mimic the function of hu CD59 and C9 can be identified using rational drug design. The compounds preferably include
30 the surface active functional groups of hu CD59 or C9, or substantially

similar groups, in the same or substantially similar orientation, so that the compounds possess the same or similar biological activity. The surface active functional groups in CD59 and C9 possess a certain orientation when they are in their active conformations, in part due to their secondary or tertiary structure. Rational drug design involves both the identification and chemical modification of suitable compounds which mimic the function of the parent molecules.

Compounds that mimic the conformation and desirable features of a particular peptide, e.g., an oligopeptide, but that avoid undesirable features, e.g., flexibility (loss of conformation) and metabolic degradation, are known as "peptidomimetics". Peptidomimetics that have physical conformations which mimic the three dimensional structure of amino acids 42-58, in particular, which have surface active groups as described herein, are active in inhibiting the formation of the C5b-9 complex. Peptidomimetics that have physical conformations which mimic the three dimensional structure of amino acids 359-384 of hu C9 are active in blocking complement inhibition.

The physical conformation of hu CD59 and C9 are determined, in part, by their primary, secondary and tertiary structure. The primary structure of a peptide is defined by the number and precise sequence of amino acids in CD59 or C9. The secondary structure is defined by the extent to which the polypeptide chains possess any helical or other stable structure. The tertiary structure is defined by the tendency for the polypeptides to undergo extensive coiling or folding to produce a complex, somewhat rigid three-dimensional structure.

1. Computer Modeling Software

Computer modeling technology allows visualization of the three-dimensional atomic structure of a selected molecule and the rational design of new compounds which will mimic the molecule or which will interact with the molecule. The three-dimensional structure can be determined based on data from x-ray crystallographic analyses and/or NMR imaging of the

selected molecule, or from *ab initio* techniques based solely or in part on the primary structure, as described, for example, in U.S. Patent No. 5,612,895 to Balaji et al. The computer graphics systems enable one to predict how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity.

- Many databases and computer software programs are known that can be used to design drugs. For example, see Ghoshal et al., "Computer Aids in Drug Design - Highlights" (1996) *Pol. J. Pharmacol.* 48(4), 359-377;
- 10 Wendoloski et al., "Biophysical Tools for Structure-Based Drug Design" (1993) *Pharmacol. Ther.* 60(2), 169-183; Lybrand, "Ligand-Protein Docking and Rational Drug Design" (1995) *Curr. Opin. Struct. Biol.* 5(2), 224-228; Kleinberg and Wanke, "New Approaches and Technologies in Drug Design and Discovery" (1995) *Am. J. Health Syst. Pharm.* 52(12), 1323-1336;
- 15 Kubinyi, "Strategies and Recent Technologies in Drug Discovery" (1995) *Pharmazie* 50(10), 647-662; Archakov et al., (1996) *Vestn. Ross. Akad. Med. Nauk.* 1, 60-63; Taylor and Smith, "The Word Wide Web as a Graphical User Interface to Program Macros for Molecular Graphics, Molecular Modeling, and Structure-Based Drug Design" (1996) *J. Mol.*
- 20 *Graph.* 14(5), 291-296; Huang et al., "Development of a Common 3D Pharmacophore for Delta-Opioid Recognition From Peptides and Non-Peptides Using a Novel Computer Program" (1997) *J. Comput. Aided Mol. Des.* 11(1), 21-78; and Li et al., "A computer Screening Approach to Immunoglobulin Superfamily Structures and Interactions: Discovery of Small
- 25 Non-Peptidic CD4 Inhibitors and Novel Immunotherapeutics (1997) *Proc. Natl. Acad. Sci. USA* 94(1), 73-78.

Data bases including constrained metabolically stable non-peptide moieties may be used to search for and to suggest suitable CD59 or C9 analogs. Searches can be performed using a three dimensional data base for

non-peptide (organic) structures (e.g., non-peptide analogs, and/or dipeptide analogs) having three dimensional similarity to the known structure of the active regions of these molecules. See, e.g., the Cambridge Crystal Structure Data Base, Crystallographic Data Center, Lensfield Road, Cambridge, CB2 1EW, England; and Allen, F. H., et al., *Acta Crystallogr.*, B35: 2331-2339 (1979). Alternatively, three dimensional structures generated by other means such as molecular mechanics can be consulted. See, e.g., Burkert, et al., *Molecular Mechanics*, American Chemical Society, Washington, D.C. (1982); and Weiner, et al., *J. Am. Chem. Soc.*, 106(3): 765-84 (Eng.) (1984).

Search algorithms for three dimensional data base comparisons are available in the literature. See, e.g., Cooper, et al., *J. Comput.-Aided Mol. Design*, 3: 253-259 (1989) and references cited therein; Brent, et al., *J. Comput.-Aided Mol. Design*, 2: 311-310 (1988) and references cited therein. Commercial software for such searches is also available from vendors such as Day Light Information Systems, Inc., Irvine, Calif. 92714, and Molecular Design Limited, 2132 Farallon Drive, San Leandro, Calif. 94577. The searching is done in a systematic fashion by simulating or synthesizing analogs having a substitute moiety at every residue level. Preferably, care is taken that replacement of portions of the backbone does not disturb the tertiary structure and that the side chain substitutions are compatible to retain the CD59/C9 interactions.

2. Structural Data to be Used with the Modeling Software

The chimeric studies described herein have determined which amino acids are present in the active binding region in both hu CD59 and C9. With respect to CD59, the active surface exposed side chains that are available to bind C8/C9 were identified from the solution structure of hu CD59, as determined from published NMR data and the knowledge of the active portion of the CD59 molecule. These side chains are the side chains of

histidine at position 44, asparagine at position 48, aspartic acid at position 49, threonine at positions 51 and 52, arginine at position 55 and glutamic acid at position 58. Accordingly, the receptor geometry and active conformation of the active region in hu CD59 is known, by virtue of existing NMR (Nuclear Magnetic Resonance) data. Using the information regarding bond angles and spatial geometry of the critical amino acids, one can use computer programs as described herein to develop peptidomimetics.

Chemically modified analogs of the active portion of hu C9 can also be identified using the techniques described above.

3. Chemical Modifications

Peptidomimetics can be modified to increase bioavailability. Preferably, the compounds are structurally constrained such that the surface active groups are oriented in the active conformation. The compounds can further include chemical modifications that minimize the metabolic degradation of the compounds once they are administered. See, for example, Spatola, A. F. *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins* (Weistein, B, Ed.), Vol. 7, pp. 257-357, Marcel Dekker, New York (1983), which describes the use of the methylenethio bioisostere [CH_2S] as an amide replacement; and Szelke et al., *In Peptides: Structure and Function, Proceedings of the Eighth American Peptide Symposium*, (Hruby and Rich, Eds.); pp. 579-582, Pierce Chemical Co., Rockford, Ill. (1983), which describes methyleneamino [CH_2NH] and hydroxyethylene [CHOHCH_2] bioisosteres.

CD59 and C9 have flexible and rigid portions. The flexible portions of the structure can be replaced with suitable bioisosteres or equivalents, so that the active conformation can be maintained. As defined herein, the term "bioisostere" refers to atoms or groups of atoms which are of similar size to the atom or group of atoms which are to be replaced, wherein the compound containing the replacement atom or group of atoms retains, to a substantial degree, the biological activity of the original, unmodified peptide. See, for

example, Nelson, Mautner, and Kuntz, at pp. 227, 271 and 285, respectively, in *Burger's Medicinal Chemistry, Part 1, the Basis of Medicinal Chemistry*, 4th Edition, M. E. Wolff, ed. (John Wiley & Sons, NY, 1980).

5 Numerous peptide backbone substitutions are known to those of skill in the art which can provide peptidomimetics with improved physical and chemical properties, including enhanced rigidity and chemical and/or metabolic stability. Suitable substitutions include modifying one or more of the amide bonds by replacing the amide nitrogen with an oxygen atom, or a sulfur atom, or by replacing H at the amide nitrogen with an alkyl, aryl, 10 aralkyl or alkaryl group, producing an N-substituted amide, or by replacing the amide group with a methylene moiety, optionally substituted with one or two alkyl, aryl, aralkyl or alkaryl groups, which can in turn optionally be substituted with various functional groups, such as halogens, carbonyl groups, amines, nitriles, azides, thiols, hydroxy groups, and carboxylic acid 15 groups. The alkyl groups are preferably C₁₋₆ straight, branched or cyclic groups. Further, one or more of the amide bonds present in the peptide backbone can be modified, for example, by replacing the amide carbonyl group with a methylene group (optionally substituted as described above), a thiocarbonyl group, a sulfone moiety or a sulfoxide moiety.

20 The peptide can be further modified by introducing alkyl, aryl, aralkyl or alkaryl substituents, optionally substituted as described above, at one or more of the alpha-carbon atoms, such that the peptide backbone is unchanged, but additional side chain substituents are present in the chemically modified analog. Suitable α -carbon atom modifications include 25 cyclopropyl groups, ethylidene groups, and primary, secondary or tertiary amines.

Each of these modifications can be introduced into the peptide chain in either orientation (i.e., in the orientation shown, or in the "reverse" orientation). In addition, various substituents on the amide nitrogen and the 30 α -carbon can be bound to one another, thereby forming a cyclic structure

which is a relatively constrained analog. Other constrained, cyclic structures can also be prepared by linking various substituents to form cyclic structures using chemical techniques known to those of skill in the art. Other modifications include those described in U.S. Patent No. 5,612,895 to Balaji et al., the contents of which are hereby incorporated by reference.

Chemically modified analogs are typically more resistant to enzymatic cleavage than the native peptides from which they are derived because the modified residues are not typically recognized by the enzymes which degrade naturally occurring proteins. Further, the backbone and side chains of peptides can be modified to provide peptidomimetics with reduced conformational flexibility. Accordingly, the possibility that the peptide will adopt conformation(s) other than the specifically desired conformation(s) can be substantially minimized by appropriate modification.

Methods of Preparing the Compounds

Once the desired analog (including backbone and side chain modifications, as appropriate) has been identified, chemical synthesis is undertaken, employing standard synthetic techniques. For a given target compound, the skilled artisan can readily identify suitable synthetic approaches for the preparation of the target compound. Particular techniques for synthesizing certain classes of compounds are described in more detail below.

1. Methods of Preparing Nucleotide Molecules

Nucleotide molecules which bind amino acids 42-58 of hu CD59 can be generated *in vitro*, and then inserted into cells. Oligonucleotides can be synthesized on an automated synthesizer (e.g., Model 8700 automated synthesizer of Milligen-Bioscience, Burlington, MA or ABI Model 380B). (see, e.g., Offensperger et al., 1993 EMBO J. 12, 1257-1262 (*in vivo* inhibition of duck hepatitis B viral replication and gene expression by antisense phosphorothioate oligodeoxynucleotides); Rosenberg et al., PCT WO 93/01286 (synthesis of sulfurthioate oligonucleotides); Agrawal et al.,

1988 Proc. Natl. Acad. Sci. USA 85, 7079-7083 (synthesis of antisense oligonucleoside phosphoramidates and phosphorothioates to inhibit replication of human immunodeficiency virus-1); Sarin et al., 1989 Proc. Natl. Acad. Sci. USA 85, 7448-7794 (synthesis of antisense methylphosphonate oligonucleotides); Shaw et al., 1991 Nucleic Acids Res 19, 747-750 (synthesis of 3' exonuclease-resistant oligonucleotides containing 3' terminal phosphoroamidate modifications).

To reduce susceptibility to intracellular degradation, for example by 3' exonucleases, a free amine can be introduced to a 3' terminal hydroxyl group of oligonucleotides without loss of sequence binding specificity (Orson et al., 1991). Furthermore, more stable triplexes are formed if any cytosines that may be present in the oligonucleotide are methylated, and also if an intercalating agent, such as an acridine derivative, is covalently attached to a 5' terminal phosphate (e.g., via a pentamethylene bridge); again without loss of sequence specificity (Maher et al., (1989); Grigoriev et al., 1992). Other nucleoside modifications that reduce susceptibility to intracellular degradation are well known to those of skill in the art, and are intended to be within the scope of the compositions and methods described herein.

Methods to produce or synthesize oligonucleotides are well known in the art. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see e.g., Sambrook et al., Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (see also, Ikuta et al., in Ann. Rev. Biochem. 1984 53, 323-356 (phosphotriester and phosphite-triester methods); Narang et al., in Methods Enzymol., 65, 610-620 (1980) (phosphotriester method).

2. Preparation of Peptides

Proteins can be expressed recombinantly or naturally and cleaved by enzymatic digest, expressed from a sequence encoding just a peptide, or synthesized using standard techniques. It is a routine matter to make

appropriate peptides, test for binding, and then utilize the peptides. The peptides are easily prepared by standard techniques. They can also be modified to increase *in vivo* half-life, by chemical modification of the amino acids or by attachment to a carrier molecule or inert substrate, as discussed
5 above. The peptides can also be conjugated to a carrier protein by its N-terminal cysteine by standard procedures such as the commercial Inject kit from Pierce Chemicals or expressed as a fusion protein, which may have increased stability. Solid phase synthesis described by J. Merrifield, 1964 J. Am. Chem. Soc. 85, 2149, used in U.S. Patent No. 4,792,525, and
10 described in U. S. Patent No. 4,244,946, wherein a protected alpha-amino acid is coupled to a suitable resin, to initiate synthesis of a peptide starting from the C-terminus of the peptide. Other methods of synthesis are described in U.S. Patent No. 4,305,872 and 4,316,891, the contents of which are hereby incorporated by reference. These methods can be used to
15 synthesize peptides having identical sequence to the receptor proteins described herein, or substitutions or additions of amino acids, which can be screened for activity as described above.

The peptide can also be prepared as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as
20 hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium
25 hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

Peptides containing cyclopropyl amino acids, or amino acids
derivatized in a similar fashion, can also be used. These peptides retain their original activity but have increased half-lives *in vivo*. Methods known for

modifying amino acids, and their use, are known to those skilled in the art, for example, as described in U.S. Patent No. 4,629,784 to Stammer.

Methods for Evaluating the Compounds for Biological Activity

After the compounds are synthesized, their biological activity can be evaluated, for example, using competitive binding studies, and iterative refinement of the peptidomimetic (in the case of a constrained analog itself) can then be carried out. Those chemically modified analogs which are biologically active can be employed as peptidomimetics without further modification.

10 Compositions Including the Compounds

The compounds described above are preferably administered in a pharmaceutically acceptable vehicle. Suitable pharmaceutical vehicles are known to those skilled in the art. For parenteral administration, the compound will usually be dissolved or suspended in sterile water or saline.

15 The compounds can also be administered locally by topical application of a solution, cream, gel, or polymeric material (for example, a Pluronic™, BASF). Alternatively, the compound may be administered in liposomes or microspheres (or microparticles). Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled in the art. U.S. Patent No. 4,789,734 describe methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary.

20 A review of known methods is by G. Gregoriadis, Chapter 14. "Liposomes", Drug Carriers in Biology and Medicine pp. 287-341 (Academic Press, 1979). Microspheres formed of polymers or proteins are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the bloodstream. Alternatively, the compound can be incorporated and the microspheres, or composite of

30 microspheres, implanted for slow release over a period of time, ranging from

days to months. See, for example, U.S. Patent No. 4,906,474, 4,925,673, and 3,625,214, the contents of which are hereby incorporated by reference.

Methods of Treatment

5 An effective amount of the compositions described above is that which achieves the desired effect: either to inhibit assembly of the C5b-9 complex by binding to C9 or to bind to the endogenous CD59 to prevent the CD59 from inhibiting assembly of the C5b-9 complex, thereby increasing complement-mediated activation, injury or cytolysis of cells.

10 The peptides are generally active when administered parenterally in amounts above about 1 μ g/kg of body weight. Based on extrapolation from other proteins, for treatment of most inflammatory disorders, the dosage range will be between 0.1 to 70 mg/kg of body weight. This dosage will be dependent, in part, on whether one or more peptides are administered. Based on studies with other peptide fragments blocking binding, the IC₅₀, the
15 dose of peptide required to inhibit binding by 50%, ranges from about 50 μ M to about 300 μ M, depending on the peptides. These ranges are well within the effective concentrations for the *in vivo* administration of peptides, based on comparison with the RGD-containing peptides, described, for example, in U.S. Patent No. 4,792,525 to Ruoshalaghti, et al., used *in vivo* to alter cell
20 attachment and phagocytosis.

Inhibition of C5b-9 complex assembly is useful for all disorders characterized by excessive complement activation or complement-mediated cytolysis, including, for example, immune disorders and diseases such as immunovasculitis, rheumatoid arthritis, scleroderma, disseminated
25 intravascular coagulation, lupus, paroxysmal nocturnal hemoglobinuria, thrombotic thrombolytic purpura, vascular occlusion, reocclusion after surgery, coronary thrombosis, and myocardial infarction. Inhibition of CD59 is useful as an adjuvant for tumor therapy and as a contraceptive since its been demonstrated that CD59 protects sperm from rejection by antibody and

complement in the female genital tract and that CD59 expressed on human tumor cells protect these cells from complement-mediated lysis.

Recent evidence suggests that complement inhibitors specifically directed against hu MAC have potential clinical use in preventing hyperacute rejection of transplanted organs and in reducing the pathological consequences of complement activation in various immune and inflammatory diseases. Identification of the specific protein motif that is responsible for the selective inhibitory action of CD59 towards the pore-forming and cytolytic properties of hu MAC allows one to rationally design small molecules that can mimic the protective effect of this natural cell-surface complement inhibitor.

The present invention will be further understood by reference to the following studies.

Example 1: Demonstration of role of amino acids 42-58 within hu CD59 in the species-selectivity of CD59.

EXPERIMENTAL PROCEDURES

Materials – Rabbit whole blood and chicken whole blood in ACD were from Cocalico Biologics, Inc. (Reamstown, PA). Human serum, rabbit serum, human serum depleted of complement protein C8 (C8D), human complement proteins (C5b7, C8 and C9) and rabbit complement proteins C8 and C9 were purified and assayed as described previously (Hüsler, T., Lockert, D. H., Kaufman, K. M., Sodetz, J. M., and Sims, P. J. (1995) *J. Biol. Chem.* 270, 3483-3486; Rollins, S. A. and Sims, P. J. (1990) *J. Immunol.* 144, 3478-3483; Wiedmer, T. and Sims, P. J. (1985) *J. Membr. Biol.* 84, 249-25827; and Wiedmer, T. and Sims, P. J. (1985) *J. Biol. Chem.* 260, 8014-8019). 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulphonate (CHAPS), Phenyl methyl sulfonyl fluoride (PMSF), Dimethyl

5 sulfoxide (DMSO) and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Nonidet P-40, and Triton X-100 were from CalBiochem., Inc. (La Jolla, CA). Polyoxyethylene 20-soritan monolaurate (Tween 20) was from Fisher Chemical (Fairlawn, NJ). TA cloning kit, mRNA purification kit, Escherichia coli (E. coli) strain TOP10 and pcDNA3 vectors were obtained from Invitrogen (San Diego, CA). All restriction endonucleases were from New England Biolab (Beverly, MA). T4 ligase, Hank's balanced salt solution (HBSS), Dulbecco's modified eagle's medium (DMEM) and DNA primers were purchased or synthesized in GIBCO BRL life Technologies (Gaithersburg, MD). Wizard DNA purification kit was from Promega (Madison, WI). Advanced KlenTaq enzymes, cDNA library construction kit, Marathon racing kit was from Clontech (Palo Alto, CA). Sequenase version 2.0 kit was from Amersham/USB (Cleveland, OH). SV-T2 cell line (ATCC163.7) was obtained from American Type Culture Collection (Rockville, MD). BCECF-AM dye was purchased from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS), calf serum, cell dissociation buffer, L-glutamate, penicillin, streptomycin, trypsin and geneticin were from Sigma. FITC-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratory (West grove, PA). Rabbit anti-mouse lymphocyte IgG was the product of Inter-Cell Technologies (Hopewell, NJ). Hu CD59 cDNA-PUC18 and murine monoclonal antibody (mab) 9E10 against TAG peptide were generous gifts from Dr. A. L. M. Bothwell (Yale Medical School, New Haven, CT). Silver stain, Coomassie and BCA protein assay reagents were from Pierce (Rockford, IL). All other chemicals were reagent grade or better.

Isolation of rabbit CD59 – Rabbit erythrocyte ghost membranes were prepared as described for hu CD59 purification, and suspended to a final volume representing 1.5 times the original volume of packed erythrocytes (Davies et al., (1989) *J. Exp. Med.* 170, 637-654). The ghost suspension was brought to 150 mM NaCl, 1 mM phenylmethyl sulfony fluoride, and 1-

butanol added slowly to 20% vol/vol. Following stirring (3 hr at 4°C) and centrifugation (30 min, 4°C at 10,000 x g), the butanol-saturated aqueous phase was collected, CHAPS added to final 0.1% (v/v), and dialyzed against 20 mM Tris, 0.1% CHAPS, pH 7.4. The dialyzed extract was applied to

5 2.5 X 10-cm DEAE Sepharose Fast Flow column (Sigma) equilibrated in the same buffer and eluted with 500 ml of linear NaCl gradient (0-400 mM). Fractions were tested for MAC inhibitory function, using chicken erythrocyte targets cells as described previously, substituting rb C8 and C9 for hu C8 and C9 (Rollins, S. A. and Sims, P. J. (1990) *J. Immunol.* 144, 3478-3483).

10 Fractions containing rb MAC inhibitory activity were pooled and NaCl added to final concentration of 300 mM. The pool was applied to 1.6 X 8-cm phenyl-Sepharose™ column (Sigma) equilibrated with 300 mM NaCl, 0.05% CHAPS, 20 mM Tris, pH 8.0. Following washing with the same solution, protein was eluted with a linear gradient representing 0.05% to 1% CHAPS,

15 300 mM to 0 mM NaCl, in 20 mM Tris, pH 8.0. The active fractions were pooled and further purified on 0.5 X 5-cm Mono Q HR™ column (Pharmacia Biotech Inc., Uppsala, Sweden) using a gradient of 0 to 400 mM NaCl in 0.5% CHAPS, 20 mM Tris, pH 7.4. Active fractions were concentrated by step elution on Mono Q™ and further purified by SDS PAGE using a 10%

20 NuPAGE™ gel (Novex, San Diego, CA) run under non-reducing conditions. The protein band at 20 kDa (approximately 8 µg total protein from original 300 ml packed rabbit erythrocytes) was eluted from the gel slice into 0.1% CHAPS, 20 mM Tris, pH 7.4 and inhibitory activity of the eluted protein towards rb MAC confirmed by functional assay. All column chromatography

25 procedures were performed at room temperature on a BioCAD™ 20 perfusion chromatography workstation (PerSeptive Biosystems, Framingham, MA). N-terminal sequence was then obtained through 40 cycles of Edmann degradation (Protein and Carbohydrate Structure Facility, Univ. of Michigan, Ann Arbor, MI), yielding S-L-M-C-Y-H-C-L-L-P-S-P-N-C-S-T-V-T-N-C-T-

30 P-N-H-D-A-C-L-T-A-V-S-G-P-R-V-Y-R-Q-C- (Sequence ID No. 16).

Cloning of rabbit CD59 cDNA – Degenerate oligonucleotides were

constructed based on peptide sequence and used to amplify a rabbit lymphocyte cDNA library (5'RACE, MARATHON™ Kit, CLONTECH) from which a 200 bp PCR product was obtained. Specific primers based on this 200 bp cDNA were designed and used to amplify the rabbit lymphocyte cDNA library by 3' RACE. Full-length cDNA of rb CD59 was obtained by linking the PCR products from 5' and 3' RACE using PCR. The sequence of this cDNA clone was deposited at GenBank (Accession number: AF040387) and the deduced amino acid sequence of the predicted ORF is shown in Figure 1. The predicted translation product consists of 124 residues, including a 24-residue signal peptide before the N-terminal Ser¹ of the mature protein. The unusual N-terminal Ser¹ of the mature rb CD59 protein was confirmed at both protein and DNA levels (Figure 1).

Construction of plasmids encoding rabbit/human CD59 chimeras –

The 467 bp insert encoding hu CD59 was released from PUC18 using ECORI restriction sites and subcloned into the ECORI site in pcDNA3 expression vector. The vector with correct CD59 cDNA orientation was selected and used as a template for PCR. A 33 bp oligonucleotide (corresponding to the TAG peptide sequence EQKLISEEDLN (Sequence ID No. 17)) was inserted between the leader sequence of CD59 and the N-terminal amino acid (Leu¹) of the mature protein using PCR. Rb CD59-TAG in pcDNA3 vector was made by replacing the sequence in hu CD59-TAG pcDNA3 with the sequence encoding mature rb CD59 and the rb CD59 C-terminal signal using HindIII and XbaI sites in pcDNA3. cDNAs encoding the chimeric hu/rb CD59 constructs depicted in Figure 2 were prepared using PCR amplification by procedures previously described by Zhou, Q., Zhao, J., Hüsler, T., and Sims, P. J. (1996) *Mol. Immunol.* 33, 1127-1134. The pcDNA3 plasmids containing hu CD59 sequence or rb CD59 sequence were used as templates to generate the cDNA encoding chimeric CD59 proteins. The chimeric cDNA was then inserted into pcDNA3 vector using HindIII and

XbaI sites. Hu, rb, and hu/rb chimeric CD59-TAG pcDNA3 plasmids were used to transform *E. coli* strain TOP10. Constructs from independent colonies were sequenced in their entirety in both directions by automated DNA sequencing (Applied Biosystems, Inc.) or by dideoxy-sequencing using a sequenase version 2.0 kit. Plasmids containing the desired constructs without nucleotide error were selected and amplified for expression in the SV-T2 cell line.

Expression of CD59 constructs in SV-T2 cells – SV-T2 cells were transfected with hu, rb, or chimeric TAG-CD59 pcDNA3 by electroporation as previously described (Zhou, Q., Zhao, J., Hüsler, T., and Sims, P. J. (1996) *Mol. Immunol.* 33, 1127-1134). After 48 hr, stable transfectants were selected with DMEM complete medium containing 1 mg/ml geneticin for 10 days. If episomal replication in the transfected cells was desired, 8 X 10⁶ SV-T2 cells were transfected with 120 µg plasmid DNA by electroporation using Gene Pulse (Bio-rad) at 360 V and 500 µF (Zhou, Q., Zhao, J., Hüsler, T., and Sims, P. J. (1996) *Mol. Immunol.* 33, 1127-1134). Geneticin-selected cells were stained with mAb 9E10 against TAG epitope followed by FITC-conjugated goat antibody against mouse IgG, and sorted by flow cytometry (FACStar Plus; Becton Dickinson). Individual clones were then obtained by limiting dilution in DMEM containing 0.5 mg/ml geneticin. Comparison was made to clonal cell lines derived by transfection with pcDNA3, lacking insert (vector-only controls).

Measurement of cell surface TAG-CD59 – The cell surface expression of each TAG-CD59 construct in transfected SV-T2 cells was quantified by the binding at saturation of mAb 9E10 (against TAG epitope) as previously described by Zhou, Q., Zhao, J., Hüsler, T., and Sims, P. J. (1996) *Mol. Immunol.* 33, 1127-1134. Following growth to near confluence, cells were detached and incubated for 30 min at 23°C with mAb 9E10 (100 µg/ml) in HBSS containing 1 % BSA. After washing with HBSS containing 1 % BSA,

the cells were incubated (20 min at 23°C) with FITC conjugated goat anti-mouse IgG at a final concentration of 10 µg/ml. The fluorescence was determined by flow cytometry (FACScan, Becton Dickinson) as described previously by Zhou, Q., Zhao, J., Hüsler, T., and Sims, P. J. (1996) *Mol.*

5 *Immunol.* 33, 1127-1134.

Assay of MAC inhibitory function - The complement-inhibitory activity of recombinant CD59 expressed on the transfected SV-T2 cells was evaluated by minor modification of methods previously described (Zhou, Q., Zhao, J., Hüsler, T., and Sims, P. J. (1996) *Mol. Immunol.* 33, 1127-1134, and Zhao, J., Rollins, S. A., Maher, S. E., Bothwell, A. L., and Sims, P. J. (1991) *J. Biol. Chem.* 266, 13418-13422). For cell clones expressing each chimeric CD59 construct, huC5b67 was deposited on the plasma membrane and susceptibility to the lytic activity of either hu C8 and C9 or rb C8 and C9 was measured and compared to identically-treated clones expressing wild-
15 type hu or rb CD59. Briefly, SV-T2 cells grown to 80% confluence were washed and loaded with BCECF-AM dye. C5b67 complexes were deposited on the cells using 40% hu C8D serum as complement source. After two washes, the C5b67 cells were incubated in serum-free medium with either 2 µM hu C8 and 5 µM hu C9, or with 4 µM rb C8 and 5 µM rb C9. MAC-
20 mediated cell lysis was determined from the measured release of BCECF dye from the cytoplasm, with correction for non-specific dye leak from matched controls omitting C8 and C9, as previously described by Zhou, Q., Zhao, J., Hüsler, T., and Sims, P. J. (1996) *Mol. Immunol.* 33, 1127-1134. Under these conditions, MAC-mediated lysis of the vector-only SV-T2 controls not
25 expressing recombinant CD59 ranged from 75-90%.

RESULTS

Cloning of rabbit CD59 - The predicted translation product of cDNA encoding rb CD59 consists of 124 residues, including a 24-residue signal peptide, a predicted GPI attachment site, and a 23-residue signal peptide

including a transmembrane domain C-terminal to the predicted transamidase cut site (Figure 1) (Kinoshita, T., Inoue, N., and Takeda, J. (1995) *Adv. Immunol.* 60, 57-103, and Gerber, L. D., Kodukula, K., and Udenfriend, S. (1992) *J. Biol. Chem.* 267, 12168-12173). N-terminal sequencing of protein purified from rabbit erythrocytes and analysis of the signal peptidase cleavage site of the translated cDNA, confirmed that rb CD59 contains an additional Ser residue before the highly-conserved N-terminal Leu¹ found in all other CD59 homologues that have been sequenced to date. Therefore, in order to simplify discussion of the aligned residues of hu and rb CD59 in various chimeric constructs, residues of the mature rb CD59 polypeptide have been renumbered commencing with [N-Ser⁰]-Leu¹-Met²-Cys³- etc. (see Figure 1). All references to amino acids in rb C59 are based on this re-numbering of residues in the mature polypeptide. Whereas Gly⁷⁶ is a predicted transamidase cut site for GPI attachment, the possibility of cleavage at another residue (e.g., Asp⁷⁴) cannot be excluded.

Species-selective activity of human and rabbit CD59 – SV-T2 cell lines expressing various levels of cell surface CD59 (hu or rb) were produced through stable transfection with plasmid pcDNA3-TAG-CD59. Each cell line was then tested for its capacity to resist lysis by C5b-9. As has been previously described, cells transfected to express hu CD59 were nearly completely protected from lysis by hu C5b-9 and this protective effect of hu CD59 was not observed when rb C8 and C9 substituted for hu C8 and C9 in the C5b-9 complex (plotted curves in ^{Figures 2B-C} ~~Figure 2~~). On the other hand, rb CD59 expressed on the surface of this murine cell line conferred a selective resistance to lysis by C5b-9 assembled from rb C8 and C9, whereas virtually no inhibition of the lytic action of MAC was observed when hu C5b-9 components were used. These data confirm that recombinant rb CD59 shows the same homologous species-selective complement inhibitory function as was inferred from the differential susceptibility of rabbit erythrocytes to lysis by

human *versus* rabbit complement (Houle, J. J. and Hoffmann, E. M. (1984) *J. Immunol.* 133, 1444-1452, and Houle, J. J., Hoffmann, E. M., and Esser, A. F. (1988) *Blood* 71, 287-292). As previously noted for the TAG-huCD59 construct (Zhou, Q., Zhao, J., Hüsler, T., and Sims, P. J. (1996) *Mol.*

- 5 *Immunol.* 33, 1127-1134), these data also suggest that the TAG-rbCD59 fusion protein retains the properties of native rb CD59 that is expressed in the rabbit erythrocyte membrane

- Human-rabbit CD59 chimeras – In order to probe which residues of hu CD59 conferred its ability to selectively inhibit lysis by hu C5b-9,
- 10 chimeric proteins in which segments of the rb and hu CD59 polypeptides were interchanged were constructed. The choice of constructs reflected (i) identity of amino acid residues exposed on the surface of hu CD59 that were *not* conserved in the aligned polypeptide sequence of rb CD59, where selection was based on the reported solution structure of the glycosylated
- 15 protein, utilizing surface residues considered not to be occluded by the N-linked carbohydrate (Fletcher, C. M., Harrison, R. A., Lachmann, P. J., and Neuhaus, D. (1994) *Structure* 2, 185-199; Kieffer, B., Driscoll, P. C., Campbell, I. D., Willis, A. C., van der Merwe, P. A., and Davis, S. J. (1994) *Biochemistry* 33, 4471-4482; and Fletcher, C. M., Harrison, R. A.,
- 20 Lachmann, P. J., and Neuhaus, D. (1993) *Protein Sci.* 2, 2015-2027); (ii) an attempt to group these various non-conserved amino acid side chains into contiguously-clustered spatial arrays; and (iii) a consideration of prior data relating to potential identity of the active site residues in hu CD59 as deduced from peptide studies, site-directed mutagenesis in the protein, or from
- 25 analysis of other CD59 chimeras (Bodian, D. L., Davis, S. J., Morgan, B. P., and Rushmere, N. K. (1997) *J. Exp. Med.* 185, 507-516; Yu, J. H., Dong, S. H., Rushmere, N. K., Morgan, B. P., Abagyan, R., and Tomlinson, S. (1997) *Biochemistry* 36, 9423-9428; Zhou, Q., Zhao, J., Hüsler, T., and Sims, P. J. (1996) *Mol. Immunol.* 33, 1127-1134; Yu, J. H.,

Abagyan, R., Dong, S. H., Gilbert, A., Nussenzweig, V., and Tomlinson, S. (1997) *J. Exp. Med.* 185, 745-753; Petranka, J., Zhao, J., Norris, J., Tweedy, N. B., Ware, R. E., Sims, P. J., and Rosse, W. F. (1996) *Blood Cells Mol. Dis.* 22, 281-296; and Nakano, Y., Tozaki, T., Kikuta, N., Tobe, T., Oda, E., Miura, N.-H., Sakamoto, T., and Tomita, M. (1995) *Mol. Immunol.* 32, 241-247). Based upon these considerations, six hu/rb CD59 chimeras and, the six complementary rb/hu CD59 chimeras were analyzed and constructed so as to replace the surface-exposed side-chains contributed by residues (i) 8, 10, 12, and 14 (*chimeras Ch1 and Ch1R*; Figure 2A); (ii) 5, 37, and 38 (*chimeras Ch2 and Ch2R*; Figure 2B); (iii) residues 20, 21, 22, and 41 (*chimeras Ch3 and Ch3R*; Figure 2C); (iv) residues 60 and 62 (*chimeras Ch4 and Ch4R*; Figure 2D); (v) 29, 30, 60, and 62 (*chimeras Ch5 and Ch5R*; Figure 2E); or (vi) residues 44, 48, 49, 51, 52, 55, and 58 (*chimeras Ch6 and Ch6R*; Figure 2F). Individual clones expressing each of these recombinant proteins were obtained and the expression level on the cell-surface determined from the N-terminal TAG-epitope common to each construct. In each case, two separate clones were expanded for independent assay of MAC-inhibitory function.

MAC-inhibitory function of recombinant CD59 chimeras - SV-T2

clones expressing the chimeric constructs were analyzed for their capacity to restrict lysis mediated by MAC. The species selectivity of the complement inhibitory function of each construct was tested using hu *versus* rb C8 and C9 to assemble the C5b-9 complex. In each case, results for the chimeric constructs were compared to those obtained for transfected SV-T2 cells expressing full-length CD59 (hu or rb) and to vector-transfected SV-T2 cells lacking the CD59 insert (Figure 2). As the data of these figures reveal, the species-selective inhibitory function of either hu CD59 or rb CD59 was unaffected by the amino acid substitutions contained in chimeras Ch1/Ch1R, Ch2/Ch2R, Ch4/Ch4R, or Ch5/Ch5R (Figure 2A, 2B, 2D, and 2E). This

indicates that residues 1-19, 29,30, 37,38, 60, and 62 of the CD59 polypeptide do not directly contribute to its selective avidity for homologous C8 and C9. The results that were obtained for the Ch1/Ch1R chimeras were consistent with recent observations made with hu CD59/Ly6E chimeras, which had suggested that the N-terminal residues of the hu CD59 polypeptide do not contribute to its MAC-inhibitory function (see *Discussion*).

By contrast to results above, substitution of hu CD59 residues 42-58 into rb CD59 resulted in a protein (*chimera Ch6*) that was functionally indistinguishable from native hu CD59 whereas the complementary construct (rabbit 42-58 substituted in hu CD59; *chimera Ch6R*) was functionally indistinguishable from rb CD59. These data imply that the amino acid side-chains contributed by residues contained between Phe⁴²-Glu⁵⁸ in hu CD59 are responsible for the selective avidity of this complement inhibitor for hu MAC (see *Discussion*). In the case of chimeras Ch3 and Ch3R, a partial loss of CD59's complement-inhibitory function was observed. Replacement of residues 20-22 and 41 in rb CD59 with the corresponding residues from hu CD59 reduced rb CD59's inhibitory activity towards MAC assembled with rb C8/C9, but this substitution did not confer upon rb CD59 the capacity to inhibit hu MAC. Similarly, substitution of hu CD59 residues 20-22 and 41 with the corresponding residues from rb CD59 reduced hu CD59's inhibitory activity towards hu MAC, but this substitution did not confer upon hu CD59 the capacity to inhibit MAC assembled with rb C8/C9.

SUMMARY

The data indicates that residues 42-58 of hu CD59 contain the segment of the protein that is responsible for its species-restricted MAC-inhibitory function. As shown in *Figure 2F*, substitution of hu CD59 residues 42-58 into rb CD59 results in a protein that was functionally indistinguishable from hu CD59 whereas the complementary construct (rabbit 42-58 substituted into hu CD59) was functionally indistinguishable from rb

CD59. Within this portion of the polypeptide, residues 43, 45, 46, 47, 53, 54, 56 and 57 are identically conserved between human and rabbit. From the solved solution structure of hu CD59, the side-chains of residue 42 and 50 are buried. This indicates that the residues of hu CD59 which dictate its selective ability to bind to hu C8 and C9 are localized to a cluster of amino acid side-chains exposed on the surface of the protein that are contributed by His⁴⁴, Asn⁴⁸, Asp⁴⁹, Thr⁵¹, Thr⁵², Arg⁵⁵, and Glu⁵⁸. In addition to these residues, data for Ch3/Ch3R (chimeric substitution of residues at position 20, 21, 22 and 41) indicates that the side-chains of one or more of these residues can also influence the species selectivity of hu CD59. Among these residues, it is noteworthy that the side chain of Lys⁴¹ (replaced by Arg⁴¹ in rb CD59) projects in close proximity to the side-chain of His⁴⁴ (replaced by Asp⁴⁴ in rb CD59), a residue contained within the functionally dominant segment identified by the Ch6/Ch6R chimeras (cf. Figures 2C and 2E). The relatively conservative Lys→Arg substitution of a side-chain located in proximity to the functionally-dominant region of the protein, may explain why a relatively small loss of activity towards homologous MAC was observed for the Ch3/Ch3R constructs, and why this was not accompanied by a comparable gain in MAC-inhibitory function towards the heterologous complement proteins.

The residues identified in hu CD59 to confer its species-selective interaction with hu C8 α and C9 (i.e., His⁴⁴, Asn⁴⁸, Asp⁴⁹, Thr⁵¹, Thr⁵², Arg⁵⁵, and Glu⁵⁸) form a distinct cluster on the non-glycosylated surface of the protein and would presumably be available for a binding function.

Example 2: Demonstration of role of a disulfide bonded peptide loop within hu C9 in the species-selectivity of CD59.

EXPERIMENTAL PROCEDURES

- 5 *Materials* - Human complement proteins C5b6, C7, C8, and C9, and human erythrocyte membrane glycoprotein CD59 were purified and assayed as described by Davies, et al. *Immunol. Res.* 12, 258-275 (1993), Wiedmer and Sims, *J. Membr. Biol.* 84, 249-258 (1985), and Wiedmer and Sims, *J. Biol. Chem.* 260, 8014-8019 (1984). Hu C9 peptide 359-384 ([allyl-K]-
- 10 CLGYHLDVSLAFSEISVGAEFNKDD-[allyl-C, Sequence ID No. 18], BSA-conjugated hu C9 peptide 359-384, and affinity-purified rabbit IgG against hu C9 peptide 359-384 were custom ordered from Quality Controlled Biochemicals (Hopkinton, MA). Full-length cDNA for hu C9 was a generous gift from Dr. J. Tschopp (University of Lausanne, Epalinges,
- 15 Switzerland) and is described by Dupuis, et al., *Mol. Immunol.* 30, 95-100 (1993). Full length cDNA for rb C9 was isolated and cloned into pSVL as reported by Husler, et al., *J. Biol. Chem.* 270, 3483-3486 (1995). Chicken erythrocytes (chE) were from Cocalico Biologics, Inc. (Reamstown, PA); COS-7 cells were from American Tissue Culture Collection (Rockville, MD);
- 20 *E. coli* strain DH5 α and Opti-MEM I were from Life Technologies Inc. (Gaithersburg, MD), Dulbecco's Modified Eagle Medium was from Mediatech Inc. (Herndon, VA), and heat-inactivated fetal bovine serum was from Biocell (Rancho Dominguez, CA). Oligonucleotides were synthesized by the Molecular Biology Core Laboratories, Blood Research Institute.
- 25 Solutions - MBS: 150 mM NaCl, 10 mM MOPS, pH 7.4; GVBS: 150 mM NaCl, 3.3 mM sodium barbital, 0.15 mM CaCl₂, 0.5 mM MgCl₂, 0.1 % (w/v) gelatin, pH 7.4; GVBE: 150 mM NaCl, 3.3 mM sodium barbital, 10 mM EDTA, 0.1 % (w/v) gelatin, pH 7.4.
- 30 *Construction of chimeric C9 cDNA's* - cDNA's coding for hu/rb C9 chimeras were constructed essentially as described by Husler, et al. (1995).

In brief, regions of sequence identity were determined from the aligned sequences of rb and hu C9, and used as junctions for chimeric cDNA construction. Based on these alignments, primers for PCR were designed to generate defined segments of rb and hu C9 cDNA's. Primers annealing to 5'-or 3'- untranslated sequence with added Xbal (5'-end) or SacI (3'-end) recognition sites were paired with chimeric primers (28-37 bp in length) and used to generate cDNA fragments that contained the desired overlapping sequence at either the 5'-or 3'-ends. These fragments were gel purified, mixed at a 1:1 molar ratio, and used in a second amplification with primers located in the 5'-and 3'-untranslated region to produce full length chimeric C9 cDNA's. Fragments were cloned into the Xbal/SacI sites of pSVL for mammalian expression. PCR fidelity was confirmed by sequencing 3'-coding sequence in each construct, starting from the stop codon and continuing through all junctions of rabbit and human sequence. In certain cases, chimeric constructs were further modified by site directed mutagenesis.

Site Directed Mutagenesis - C9 cDNA in pSVL served as a template for site-directed mutagenesis using the *Chameleon* mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis was performed using 0.25 pmol of template plasmid, 25 pmol of mutagenic primer and 25 pmol of selection primer, the latter chosen to modify Sall, Scal, or XhoI restriction sites unique to pSVL. The resulting mutagenized plasmids were subject to a minimum of two rounds of selection by restriction digest, and then transformed in *E. coli* XL1-Blue (Stratagene) for single colony isolation and plasmid purification. In all cases, mutations were confirmed by double stranded sequencing of each purified plasmid.

Transfection of COS-7 cells - Plasmid DNA used in transfections was obtained from purification over Qiagen-tips (Qiagen Inc., Chatsworth, CA). COS-7 cells were transfected using DEAE-dextran, then cultured for 24h in Dulbecco's Modified Eagle Medium (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum, after which this medium was

replaced by Opti-MEM I (Life Technologies, Inc., Gaithersburg, MD). Cell supernatants were harvested after 48-65h, PMSF (1 mM), benzamidine (1 mM) and EDTA (10 mM) were added and the supernatants concentrated at 4°C (Centricon 30, Amicon).

5 *Immunoblotting* - C9 in the COS-7 supernatants was analyzed by quantitative dot blotting using murine monoclonal antibody P9-2T as described by Husler, et al. (1995).

Biotin-CD59 - CD59 was biotinylated by incubation (1 h, room temperature) with a 20-fold molar excess of NHS-LC-biotin in 10 mM MOPS, 0.1 % Nonidet P-40, pH 9.0 followed by exhaustive dialysis against charcoal, as described by Chang, et al. *J. Biol. Chem.* 269, 26424-26430 (1994).

Analysis of the inhibitory function of CD 59 towards recombinant C9 constructs - Hemolytic activity of each C9 construct was assayed using as target cells chE that were reconstituted with purified hu CD59, as described by Husler, et al., (1995). chE were washed extensively and suspended in GVBS, and the membrane C5b67 complex assembled by mixing cells ($1.4 \times 10^9/\text{ml}$) with C5b6 ($13 \mu\text{g}/\text{ml}$) followed by addition of C7 ($1 \mu\text{g}/\text{ml}$). After 10 min., the C5b67 chE were diluted to $1.4 \times 10^8/\text{ml}$ in GVBE and incubated (10 min. 37°C) with 0 or 750 ng/ml CD59. In each case, the final concentration of Nonidet P-40 was less than 0.002%(v/v). After washing in ice-cold GVBE, 2.8×10^8 of these cells were incubated (37°C) in a total volume of 100 μl with 1 ng rb C8 plus, between zero and 50 ng of recombinant C9, serially diluted in Opti-MEM I. Hemolysis was determined after 30 minutes at 37°C , with correction for nonspecific lysis, determined in the absence of C9. In each experiment, the inhibitory activity of CD59 towards each recombinant C9 construct was determined from the reduction in complement lysis of those cells reconstituted with CD59, versus the identically-treated cells omitting CD59, measured at the midpoint of the C9 titration (i.e., 50% hemolysis). In order to directly compare results obtained

20

25

30

in experiments performed on different days, data for each recombinant C9 construct were normalized to results obtained in each experiment with hu C9.

5 *CD59 binding to hu C9 peptide 359-384* - The specific binding of CD59 to hu C9-derived peptide 359-384 was measured by microtiter plate assay with biotin-CD59, according to modification of published methods of Chang, et al. (1994) and Husler, et al. (1995). Briefly, the BSA-peptide conjugate was adsorbed to 96 well polyvinyl microplates by overnight coating at 5 μ g/ml in 0.1 M sodium bicarbonate, pH 8.5. After blocking with 1 % (w/v) BSA, wells were washed and incubated (4 hrs., 37°C) with between 10 0.5 and 1 μ g/ml biotin-CD59. After washing, the bound biotin-CD59 was detected with Vectastain (Vector Labs, Burlingame, CA), developed by addition of p-nitrophenyl phosphate (2 mg/ml) and optical density recorded at 405 nm (VMaxMicroplate Reader, Molecular Devices, Inc.). In all experiments, correction was made for background adsorption of biotin-CD59 15 to BSA-coated wells (no peptide) and for nonspecific binding of biotin-CD59 to peptide, determined in the presence of a 20-fold excess of unlabeled CD59. As a positive control for specific binding, comparison was made in each experiment to wells coated with 2 μ g/ml hu C9. The capacity of monospecific antibody against hu C9 peptide 359-384 to compete specific 20 binding of CD59 was determined by prior incubation of the BSA-peptide-coated wells with antibody (2 hrs., between 0 and 100 μ g/ml LgG) before addition of biotin-CD59.

Inhibition of MAC lysis by antibody against hu C9 peptide 359-384 - The capacity of antibody against hu C9 peptide 359-384 to inhibit MAC was 25 determined by hemolytic assay, using the chE target cells described above, omitting CD59. In these experiments, between 0 and 1 mg/ml Fab of antibody against hu C9 peptide 359-384 (or, non-immune antibody control) was added with recombinant C9 (hu, rb, or chimeric), and complement-specific lysis determined.

30

RESULTS

C9 chimeras were constructed in which the segment of C9 corresponding to the putative CD59 binding site (residues 334-415 in hu C9; ^{Figure 9A}) were interchanged between hu and rb C9. These chimeric proteins were then tested for hemolytic activity and for their sensitivity to inhibition by membrane CD59 (Figure ^{9B} ~~A~~). Substitution of hu C9 residues 334-415 into rb C9 (chimera #1) resulted in a protein that was indistinguishable from hu C9 in its sensitivity to inhibition by CD59. Conversely, when this same segment of hu C9 was replaced by the corresponding rb C9 sequence (chimera #8), the resulting chimera was indistinguishable from rb C9 and virtually unaffected by the presence of membrane CD59. In these experiments, MAC was assembled using hu C5b67 and rb C8 so as to circumvent known inhibitory interaction of CD59 with hu C8 (Rollins, et al. *J. Immunol.* 146, 2345-2351 (1991), Ninomiya and Sims *J. Biol. Chem.* 267, 13675-13680 (1992).

As depicted in Figure ¹⁰ ~~9~~, the segment of hu C9 shown to bind CD59 is immediately C-terminal to the putative membrane-spanning domain of the protein, and corresponds to a segment of polypeptide exhibiting particularly low sequence conservation when hu C9 is aligned to C9 of rabbit or other non-primate species. The most prominent divergence of sequence occurs between two cysteines (Cys359-Cys384 in hu C9) that are conserved in the human and rabbit proteins. In hu C9, these cysteines have been shown to form an intrachain disulfide bond (below), as reported by Schaller, et al. *J. Protein Chem.* 13, 472-473 (1994).

In order to further localize the segment of hu C9 recognized by CD59 and to determine the specific contribution of residues spanning the Cys359/384 disulfide, a series of hu/rb C9 chimeras was constructed by interchanging segments of corresponding hu and rb C9 sequences internal to residues 334-415. Each of these chimeric proteins was expressed and analyzed for MAC hemolytic function, and for sensitivity to inhibition by

membrane CD59. All resulting hu/rb C9 chimeras were functionally active as determined by hemolytic titration against chE containing membrane C5b-8.

As shown in ^{Figures 9A-B}~~Figure 4~~, analysis of CD59-inhibitory activity towards each of these proteins revealed inhibition of MAC lytic activity by CD59 was unaffected by replacement of all residues N-terminal to Cys359 of hu C9 with corresponding rabbit sequence (chimera #2), whereas replacement of all residues C-terminal to residue 358 of hu C9 with corresponding rabbit sequence (chimera #3) resulted in a protein indistinguishable from rb C9 and only weakly inhibited by CD59. Consistent with the results for chimeras #1-3, substitution of hu C9 residues 359-415 into the corresponding segment of otherwise rb C9 (chimera #4) resulted in a protein that was indistinguishable from hu C9, suggesting that this polypeptide segment of hu C9 (residues 359-415) contains the binding site for CD59.

To further resolve the segment of hu C9 required for species-selective interaction with CD59, additional chimeras were constructed further truncating the segment of human sequence substituted into rb C9 (chimera #5-7). Data for these chimeras revealed that whereas human residues 359-391 conferred full recognition by CD59 (chimera #5), hu C9 residues 392-415 failed to confer any recognition by CD59 (chimera #5), hu C9 residues 392-415 failed to confer any recognition by CD59 when inserted into an otherwise rb C9 (chimera #6). Truncation of the inserted segment of hu C9 sequence from 359-391 (chimera #5) to 359-384 (chimera #7) was accompanied by a small but significant reduction in inhibition of MAC lytic activity by CD59. These results imply that CD59 directly interacts with the segment of hu C9 contained between residues 359-391, with the peptide segment spanning the intrachain Cys359/384 disulfide substantially contributing to this interaction.

CD59's interaction with hu C9 was abrogated by replacement of sequence spanning this putative CD59 recognition domain with corresponding rabbit sequence (chimeras #8-12). Replacement of hu C9 residues 334-415

with corresponding rabbit sequence (chimera #8) completely eliminated hu-selective interaction with CD59, as anticipated for results obtained for the complementary construct, chimera #1. Nevertheless, when the segment of rb-derived sequence substituted into otherwise hu C9 was further truncated, the resulting chimeras (chimeras #9-12) retained a surprising degree of sensitivity to the inhibitory effects of CD59, characteristic of hu C9. Thus substitution of rabbit sequence for the residues internal to Cys359-384 of hu C9 (chimera #12) did not significantly diminish CD59's capacity to inhibit the lytic activity of C9, while C-terminal extension of the segment of rabbit sequence to residue 415 (chimera #9) did not completely eliminate interaction with CD59. Taken together with results for chimeras #1-5, these data indicate that whereas hu C9 residues 359-391 alone are sufficient to confer recognition by CD59, segments of the polypeptide immediately flanking this segment significantly contribute to the extent to which this binding site is expressed.

The Cys359/384 disulfide in hu C9 has recently been reported to be highly labile and subject to spontaneous reduction in the native protein, as reported Hatanaka, et al., *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* 1209, 117-122 (1994). Since the data suggested that residues internal to Cys359/384 contribute in-large-part to species-selective recognition by CD59, the extent to which the CD59 recognition site in C9 is affected by disruption of this bond was examined. Mutant hu C9 was expressed with Ala substitutions at Cys359 and Cys384 and tested for hemolytic activity and for sensitivity to inhibition by CD59. As revealed by data of Figure 6, disruption of this disulfide bond did not significantly affect the hemolytic activity of the protein nor the capacity of CD59 to specifically inhibit C9 lytic activity. This suggests that the segment of hu C9 forming the CD59 binding site is either conformationally constrained independent of the Cys359-384 disulfide, or, that this binding site is expressed in the primary structure of hu C9, independent of protein folding.

In order to confirm that the peptide segment spanning hu C9 359-384 can itself mediate interaction with CD59, this 26 residue peptide was synthesized, coupled to BSA, and analyzed for CD59 binding, using biotin-CD59 conjugate in a micro plate assay. As demonstrated by Figure 1¹², biotin-CD59 specifically bound to C9 peptide 359-384, and this binding was inhibited by excess unlabeled CD59 or by antibody directed against the peptide.

CD59 is known to bind to C9 after C9 incorporates into the C5b-9 complex, and through this interaction inhibit propagation of membrane-inserted C9 polymer, limiting lytic activity of MAC. In order to confirm the importance of the peptide segment recognized by CD59 to MAC assembly, Fab of antibody raised against the hu C9 peptide 359-384 was tested for its capacity to inhibit the hemolytic activity of the hu C5b-9 complex, under the same condition used to evaluate the inhibitory function of CD59. As shown by the data of Figures 1¹³ A-D, this Fab inhibited hemolytic activity of hu C9 (Figure 1¹³ A) and C9 chimera #7 (representing rb C9 containing hu C9 residues 359-384, ~~Figure 1~~, Figure 1¹³ B), but had no effect on the hemolytic activity of either rb C9 (Figure 1¹³ C) or chimera #12 (representing substitution of the corresponding segment of rb C9 residues into hu C9; ~~Figure 1~~, Figure 1¹³ D).

The experiments show that hu C9 residues 359-391 promote CD59 binding, and that this segment of hu C9 contributes to the species-selective regulation of MAC function, providing an initial clue to the structural motif(s) through which this inhibitor selectively regulates the lytic activity of hu C5b-9 complex. These data further indicate that the capacity of CD59 to optimally interact with this segment of hu C9 is significantly influenced by residues immediately C-terminal to this segment of the C9 polypeptide.

Whereas the data establish that residues internal to Cys359-Cys384 contribute to recognition by CD59, the disulfide bond between these two Cys is apparently not required either for maintenance of C9's hemolytic activity

within MAC, or, for normal regulation of that activity by membrane CD59. These conclusions derived by Cys/Ala mutagenesis in recombinant hu C9 (Figure 6) are consistent with previous reports indicating: (i) the intrinsic liability of the Cys 359-384 disulfide in C9 purified from hu plasma, where
5 spontaneous reduction of this bond did not appear to alter C9 hemolytic activity, and (ii) that a specific CD59 binding site is retained in reduced and carboxymethylated hu C9, in hu C9-derived peptide fragments, and can be demonstrated for *E. Coli* fusion proteins contains hu C9-derived sequence spanning residues 359-384. This suggests that the CD59 binding site
10 expressed by this segment of hu C9 reflects interactions between amino acid side chains that do not require formation of the Cys 359/Cys 384 disulfide bond.

As noted above, chimeras generated by substituting limited segments of hu C9 into rb C9 revealed that the segment of hu C9 between 359-384
15 uniquely conferred recognition by CD59, and that this interaction was enhanced by C-terminal extension of human sequence to residue 391 (cf. ^{Figures 9A-B} ~~Chimeras #1-7; Figure 4~~). Surprisingly, chimeras generated by replacing these same segments of hu C9 with corresponding rb C9 sequence did not exhibit a complementary decrease in interaction with CD59, except when the
20 segment of rb-derived sequence replaced in hu C9 residues spanning 334-415 (cf. ^{Figures 9A-B} ~~Chimeras #8-12; Figure 4~~).

#9/a

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Sims, Peter J.
- (ii) TITLE OF INVENTION: Compositions and Methods to Inhibit the C5b-9 Complex of Complement
- (iii) NUMBER OF SEQUENCES: 18
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Patrea L. Pabst
 - (B) STREET: 2800 One Atlantic Center, 1201 W. Peachtree St.
 - (C) CITY: Atlanta
 - (D) STATE: GA
 - (E) COUNTRY: USA
 - (F) ZIP: 30309-3450
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US
 - (B) FILING DATE: 03-FEB-1998
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Pabst, Patrea L.
 - (B) REGISTRATION NUMBER: 31,284
 - (C) REFERENCE/DOCKET NUMBER: OMRF 170
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 404-873-8794
 - (B) TELEFAX: 404-873-8795

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 127 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Gly Ile Gln Gly Gly Ser Val Leu Phe Gly Leu Leu Leu Val Leu Ala
1           5           10           15
Val Phe Cys His Ser Gly His Ser Leu Gln Cys Tyr Asn Cys Pro Asn
20           25           30
Pro Thr Ala Asp Cys Lys Thr Ala Val Asn Cys Ser Ser Asp Phe Asp
35           40           45
Ala Cys Leu Ile Thr Lys Ala Gly Leu Gln Val Tyr Asn Lys Cys Trp
50           55           60
Lys Phe Glu His Cys Asn Phe Asn Asp Val Thr Thr Arg Leu Arg Glu
65           70           75           80
Asn Glu Leu Thr Tyr Tyr Cys Cys Lys Lys Asp Leu Cys Asn Phe Asn
85           90           95
```

Glu Gln Leu Glu Asn Gly Gly Thr Ser Leu Ser Glu Lys Thr Val Leu
 100 105 110

Leu Leu Val Thr Pro Phe Leu Ala Ala Trp Ser Leu His Pro
 115 120 125

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 124 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Rabbit
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Thr Ser Arg Gly Val His Leu Leu Leu Arg Leu Leu Phe Leu Leu
 1 5 10 15

Ala Val Phe Tyr Ser Ser Asp Ser Ser Leu Met Cys Tyr His Cys Leu
 20 25 30

Leu Pro Ser Pro Asn Cys Ser Thr Val Thr Asn Cys Thr Pro Asn His
 35 40 45

Asp Ala Cys Leu Thr Ala Val Ser Gly Pro Arg Val Tyr Arg Gln Cys
 50 55 60

Trp Arg Tyr Glu Asp Cys Asn Phe Glu Phe Ile Ser Asn Arg Leu Glu
 65 70 75 80

Glu Asn Ser Leu Lys Tyr Asn Cys Cys Arg Lys Asp Leu Cys Asn Gly
 85 90 95

Pro Glu Asp Asp Gly Thr Ala Leu Thr Gly Arg Thr Val Leu Leu Val
 100 105 110

Ala Pro Leu Leu Ala Ala Ala Arg Asn Leu Cys Leu
 115 120

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 77 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Gln Cys Tyr Asn Cys Pro Asn Pro Thr Ala Asp Cys Lys Thr Ala
 1 5 10 15

Val Asn Cys Ser Ser Asp Phe Asp Ala Cys Leu Ile Thr Lys Ala Gly
 20 25 30

Lys Lys Asp Leu Cys Asn Phe Asn Glu Gln Leu Glu Asn
65 70 75

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 77 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Owl monkey
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Gln Cys Tyr Ser Cys Pro Tyr Pro Thr Thr Gln Cys Thr Met Thr
1 5 10 15
Thr Asn Cys Thr Ser Asn Leu Asp Ser Cys Leu Ile Ala Lys Ala Gly
20 25 30
Ser Arg Val Tyr Tyr Arg Cys Trp Lys Phe Glu Asp Cys Thr Phe Ser
35 40 45
Arg Val Ser Asn Gln Leu Ser Glu Asn Glu Leu Lys Tyr Tyr Cys Cys
50 55 60
Lys Lys Asn Leu Cys Asn Phe Asn Glu Ala Leu Glu Asn
65 70 75

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 77 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Marmoset
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Gln Cys Tyr Ser Cys Pro Tyr Ser Thr Ala Arg Cys Thr Thr Thr
1 5 10 15
Thr Asn Cys Thr Ser Asn Leu Asp Ser Cys Leu Ile Ala Lys Ala Gly
20 25 30
Leu Arg Val Tyr Tyr Arg Cys Trp Lys Phe Glu Asp Cys Thr Phe Arg
35 40 45
Gln Leu Ser Asn Gln Leu Ser Glu Asn Glu Leu Lys Tyr His Cys Cys
50 55 60
Arg Glu Asn Leu Cys Asn Phe Asn Gly Ile Leu Glu Asn
65 70 75

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 75 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: peptide
 (iii) HYPOTHETICAL: NO
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu	Gln	Cys	Tyr	Asn	Cys	Ser	His	Ser	Thr	Met	Gln	Cys	Lys	Thr	Ser
1				5					10					15	
Thr	Ser	Cys	Thr	Ser	Asn	Leu	Asp	Ser	Cys	Leu	Ile	Ala	Lys	Ala	Gly
			20					25					30		
Ser	Gly	Val	Tyr	Asn	Lys	Cys	Trp	Lys	Phe	Asp	Asp	Cys	Ser	Phe	Lys
		35					40					45			
Arg	Ile	Ser	Asn	Gln	Leu	Ser	Glu	Thr	Gln	Leu	Lys	Tyr	His	Cys	Cys
	50					55					60				
Lys	Lys	Asn	Leu	Cys	Asn	Val	Asn	Lys	Gly	Ile					
65					70					75					

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 36 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: peptide
 (iii) HYPOTHETICAL: NO
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Pig
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu	Gln	Cys	Tyr	Asn	Cys	Ile	Asn	Pro	Ala	Gly	Ser	Cys	Thr	Xaa	Xaa
1				5					10					15	
Met	Asn	Cys	Ser	Tyr	Asn	Gln	Asp	Ala	Cys	Ile	Phe	Val	Xaa	Ala	Val
			20					25					30		
Pro	Pro	Lys	Thr												
			35												

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: peptide
 (iii) HYPOTHETICAL: NO
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Sheep
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu	Gln	Cys	Tyr	Ser	Cys	Ile	Asn	Gln	Val	Asp	Cys	Thr	Ser	Val	Ile
1				5				10					15		
Asn	Cys	Thr	Xaa	Asn	Gln	Asp	Ala	Cys	Leu	Tyr					

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 77 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Rabbit
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Ser Leu Met Cys Tyr His Cys Leu Leu Pro Ser Pro Asn Cys Ser Thr
1           5           10           15
Val Thr Asn Cys Thr Pro Asn His Asp Ala Cys Leu Thr Ala Val Ser
          20           25           30
Gly Pro Arg Val Tyr Arg Gln Cys Trp Arg Tyr Glu Asp Cys Asn Phe
          35           40           45
Glu Phe Ile Ser Asn Arg Leu Glu Glu Asn Ser Leu Lys Tyr Asn Cys
          50           55           60
Cys Arg Lys Asp Leu Cys Asn Gly Pro Glu Asp Asp Gly
          65           70           75

```

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 79 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Rat
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Leu Arg Cys Tyr Asn Cys Leu Asp Pro Val Ser Ser Cys Lys Thr Asn
1           5           10           15
Ser Thr Cys Ser Pro Asn Leu Asp Ala Cys Leu Val Ala Val Ser Gly
          20           25           30
Lys Gln Val Tyr Gln Gln Cys Trp Arg Phe Ser Asp Cys Asn Ala Lys
          35           40           45
Phe Ile Leu Ser Arg Leu Glu Ile Ala Asn Val Gln Tyr Arg Cys Cys
          50           55           60
Gln Ala Asp Leu Cys Asn Lys Ser Phe Glu Asp Lys Pro Asn Asn
          65           70           75

```

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 74 amino acids

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Mouse
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

Leu Thr Cys Tyr His Cys Phe Gln Pro Val Val Ser Ser Cys Asn Met
1          5          10          15
Asn Ser Thr Cys Ser Pro Asp Gln Asp Ser Cys Leu Tyr Ala Val Ala
          20          25          30
Gly Met Gln Val Tyr Gln Arg Cys Trp Lys Gln Ser Asp Cys His Gly
          35          40          45
Glu Ile Ile Met Asp Gln Leu Glu Glu Thr Lys Leu Lys Phe Arg Cys
          50          55          60
Cys Gln Phe Asn Leu Cys Asn Lys Ser Asp
          65          70

```

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 82 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```

Leu Tyr Glu Leu Ile Tyr Val Leu Asp Lys Ala Ser Met Lys Arg Lys
1          5          10          15
Gly Val Glu Leu Lys Asp Ile Lys Arg Cys Leu Gly Tyr His Leu Asp
          20          25          30
Val Ser Leu Ala Phe Ser Glu Ile Ser Val Gly Ala Glu Phe Asn Lys
          35          40          45
Asp Asp Cys Val Lys Arg Gly Glu Gly Arg Ala Val Asn Ile Thr Ser
          50          55          60
Glu Asn Leu Ile Asp Asp Val Val Ser Leu Ile Arg Gly Gly Thr Arg
          65          70          75          80
Lys Tyr

```

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 86 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Rabbit

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Arg Tyr Glu Leu Ile Tyr Val Leu Asp Lys Ala Ser Met Lys Glu Lys
1 5 10 15
Gly Ile Glu Leu Asn Asp Ile Lys Lys Cys Leu Gly Phe Asp Leu Asp
20 25 30
Leu Ser Leu Asn Ile Pro Gly Lys Ser Ala Gly Leu Ser Leu Thr Gly
35 40 45
Gln Ala Asn Lys Asn Asn Cys Leu Lys Ser Gly His Gly Asn Ala Val
50 55 60
Asn Ile Thr Arg Ala Asn Leu Ile Asp Asp Val Ile Ser Leu Ile Arg
65 70 75 80
Gly Gly Thr Gln Lys Phe
85

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ser Leu Met Cys Tyr His Cys Leu Leu Pro Ser Pro Asn Cys Ser Thr
1 5 10 15
Val Thr Asn Cys Thr Pro Asn His Asp Ala Cys Leu Thr Ala Val Ser
20 25 30
Gly Pro Arg Val Tyr Arg Gln Cys
35 40

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Lys Cys Leu Gly Tyr His Leu Asp Val Ser Leu Ala Phe Ser Glu Ile
1 5 10 15

Ser Val Gly Ala Glu Phe Asn Lys Asp Asp Cys
20 25



We claim:

1. A compound that specifically inhibits the formation of the hu C5b-9 complex selected from the group consisting of molecules structurally mimicking CD59 amino acid residues 42 to 58 when they are in a spatial orientation which inhibits formation of the hu C5b-9 complex, wherein the compound is not hu CD59.

10. A method for inhibiting C5b-9 complex assembly comprising administering to a patient in need thereof an effective amount of a composition to increase CD59 inhibition of C5b-9 complex assembly wherein the composition includes a compound selected from the group consisting of molecules structurally mimicking CD59 amino acid residues 42 to 58 which bind to C9 wherein the compound is not hu CD59.

11. The method of claim 10, wherein the compound is selected from the group consisting of proteins, peptides, nucleic acids, and small molecules which bind specifically to amino acids 359 to 384 of hu C9.

12. The method of claim 11, wherein the protein is an antibody.

13. The method of claim 11, wherein the protein is a chimeric peptide which include the amino acids 42 to 58 of the human sequence of CD59.

14. The method of claim 11, wherein the peptide is a covalently cyclized peptides comprising hu CD59 amino acid residues 42 to 58.

15. The method of claim 11, wherein the composition is a peptide of less than forty amino acids residues including amino acid residues 42 to 58 of hu CD59.

16. The method of claim 10, wherein the composition further comprises a pharmaceutically acceptable carrier for administration to patients in need thereof.

17. The method of claim 10, wherein the patient is in need of suppression of complement-mediated inflammation.

18. The method of claim 10 wherein the compound is a peptidomimetic compound comprising the side chains of hu CD59 amino acid residues His⁴⁴, Asn⁴⁸, Asp⁴⁹, Thr⁵¹, Thr⁵², Arg⁵⁵, and Glu⁵⁸ in an equivalent spacial orientation and alignment to that presented on the surface of hu CD59.

19. The method of claim 18 wherein the spacial orientation and alignment of the side chains of His⁴⁴, Asn⁴⁸, Asp⁴⁹, Thr⁵¹, Thr⁵², Arg⁵⁵, and

Glu⁵⁸ in the compound are equivalent to the spacial orientation and alignment deduced by NMR structure determination.

20. A compound that specifically promotes the formation of the hu C5b-9 complex selected from the group consisting of molecules structurally mimicking C9 amino acid residues 359 to 384 when they are in a spatial orientation which promotes formation of the C5b-9 complex, wherein the compound is not hu C9.

21. The compound of claim 20, selected from the group consisting of proteins, peptides, nucleic acids, and small molecules which bind specifically to amino acids 42 to 58 of hu CD59.

22. The compound of claim 21, wherein the protein is an antibody.

23. The compound of claim 21, wherein the protein is a chimeric peptide which includes the amino acids 359 to 384 of the human sequence of C9.

24. The compound of claim 21, wherein the peptide is a covalently cyclized peptide comprising hu C9 amino acid residues 359 to 384.

25. The compound of claim 21, wherein the composition is a peptide of less than forty amino acids residues including amino acid residues 359 to 384 of hu C9.

26. The compound of claim 20, further comprising a pharmaceutically acceptable carrier for administration to patients in need thereof.

27. A method for specifically promoting hu C5b-9 complex assembly comprising administering to a patient in need thereof an effective amount of a composition to decrease CD59 inhibition of C5b-9 complex assembly wherein the composition comprises a compound selected from the group consisting of molecules structurally mimicking C9 amino acid residues 359 to 384 when they are in a spatial orientation which promotes formation of the complex, wherein the compound is not hu C9.

28. The method of claim 27, wherein the compound is selected from the group consisting of proteins, peptides, nucleic acids, and small molecules which bind specifically to amino acids 42 to 58 of hu CD59.

29. The method of claim 28, wherein the protein is an antibody.

30. The method of claim 28, wherein the protein is a chimeric peptide which include the amino acids 359 to 384 of the human sequence of C9.

31. The method of claim 28, wherein the peptide is a covalently cyclized peptide comprising hu C9 amino acid residues 359 to 384.

32. The method of claim 28, wherein the composition is a peptide of less than forty amino acids residues including amino acid residues 359 to 384 of hu C9.

33. The method of claim 27, wherein the composition further comprises a pharmaceutically acceptable carrier for administration to patients in need thereof.

34. The method of claim 27, wherein the patient is in need of complement activation.

35. The method of claim 27, wherein the composition is administered as an adjunct to tumor therapy.

COMPOSITIONS AND METHODS TO INHIBIT
FORMATION OF THE C5b-9 COMPLEX OF COMPLEMENT

Abstract of the Invention

Compounds modulating CD59 mediated complement activity, compositions including these compounds, and methods of making and using the compounds are disclosed, which are based on the identification of the hu CD59 amino acid residues which serve as the binding site for CD59-C9 interactions. These residues correspond to amino acid residues 42-58, and bind to the region of C9 corresponding to human 334-418, more specifically, between amino acid residues 359 and 384. Compounds can be derived using this basic amino acid sequence and corresponding three dimensional structure within the protein using any of several techniques known to those skilled in the art, including rational drug design using computer data bases and modeling of peptide/protein-ligand binding, antibodies and anti-idiotypic antibodies generated to the proteins or peptides containing this peptide sequence, and modified peptides. Those compounds imitating the structure and/or function of the peptide region are referred to herein as "peptidomimetics", and include small molecules which present the surface exposed side chains in these amino acids in the same relative positions, compounds identified by combinatorial chemistry techniques which bind to the active portions of human C9, as well as modified peptides. The compounds can be used to inhibit complement by binding to C9 analogously to CD59, or to maintain complement inhibition, by blocking CD59 binding to C9. The compounds can be administered locally or systemically in any suitable carrier in an amount effective to either inhibit complement or block the inhibition of complement, in a patient in need of treatment thereof.

	-25	-20	-15	-10	-5	0	1	5	10	15
Human	GI QGGS VLFGLLVLA	VFCHSGHS	LQCYNCPNP	TADCKTAVN						
Rabbit	MTSRGVHLLRLFL	LLAVFYSS	-DSSL	MCYHCLLP	SPNCSTVTN					

	20	25	30	35	40	45	50	55	60
Human	CSSDFD	ACLI	TKAGLQVYNK	CKWKEHCN	FNDVTTRL	RENE	LTYY		
Rabbit	CTPNHDA	CLTAVSGPRVYR	QCWRVEDCN	FEFISNR	LEENSL	KYN			

	65	70	75	80	85	90	95	100
Human	CCKKDL	CNFNEQL	ENG	GGTSL	SEKTVLL	VTPFL	AAWS	LHP
Rabbit	CCRKDL	CNGPEDDG	- -	TALTGR	TVLL	-VAPLL	AAARNL	CL

FIGURE 1A

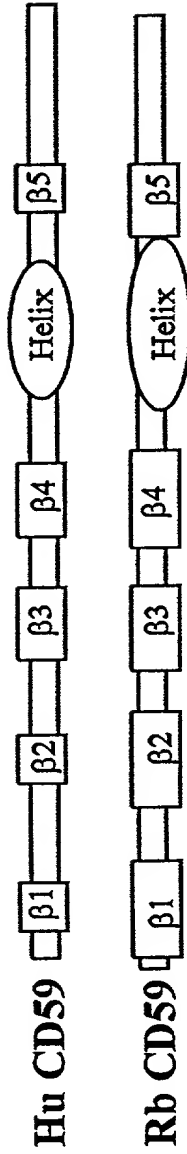
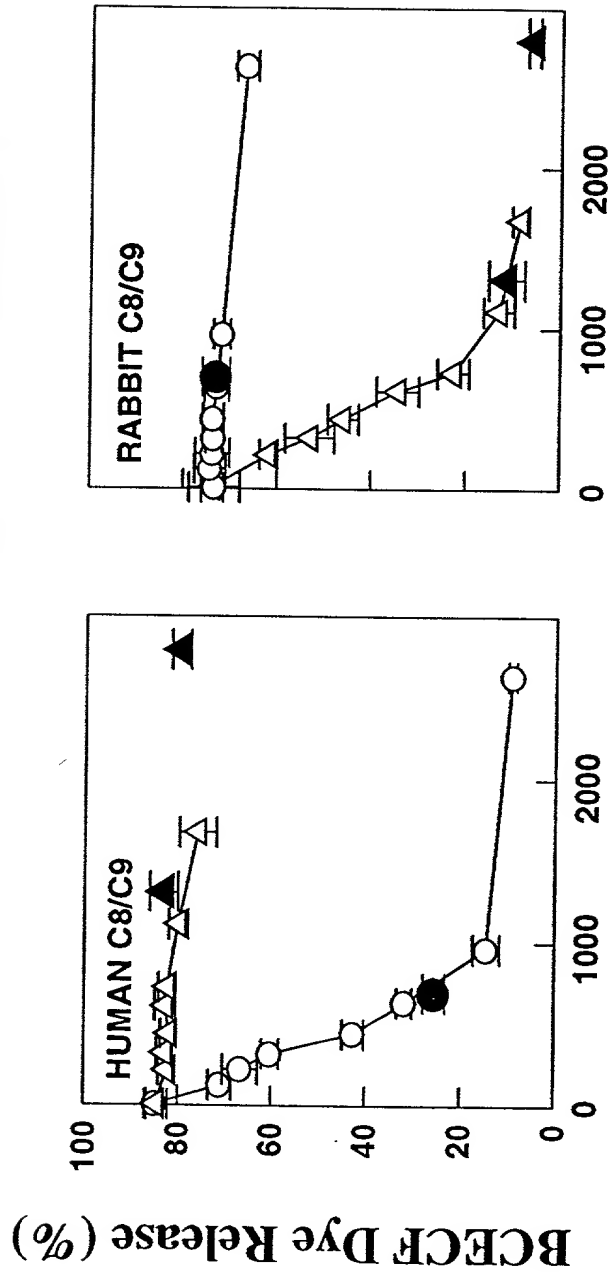
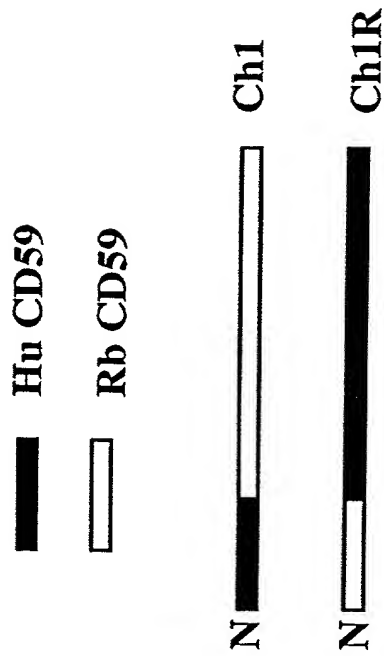


FIGURE 1B

Figure 2A

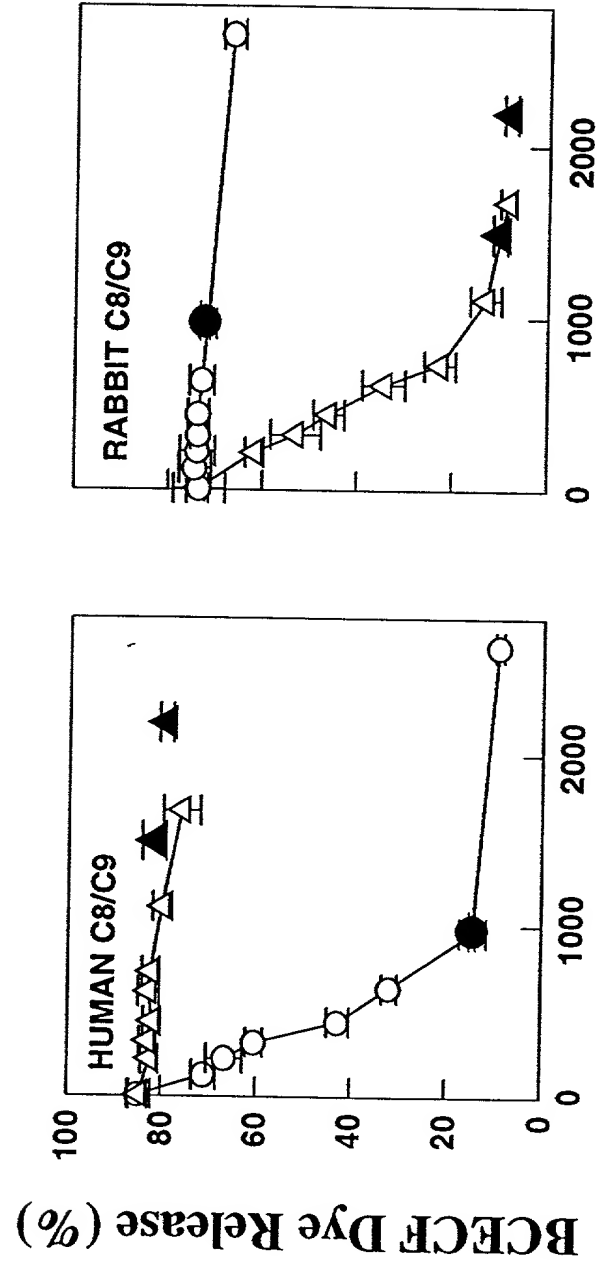
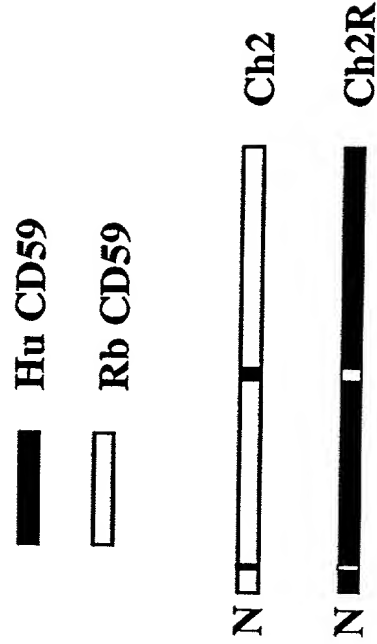


CD59 per Cell (arbitrary fluorescence units)

Figure 2B

Figure 2C



Figure 3A



CD59 per Cell (arbitrary fluorescence units)

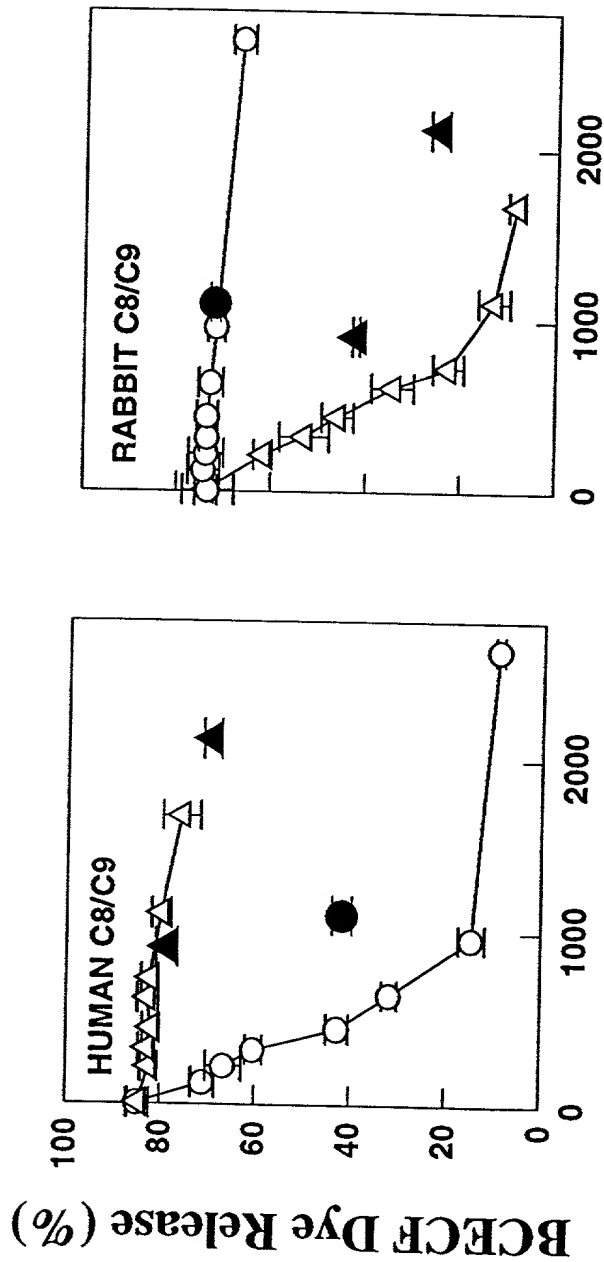
Figure 3B

Figure 3C

 Hu CD59
 Rb CD59

 N Ch3
 N Ch3R

Figure 4A



CD59 per Cell (arbitrary fluorescence units)

Figure 4B

Figure 4C

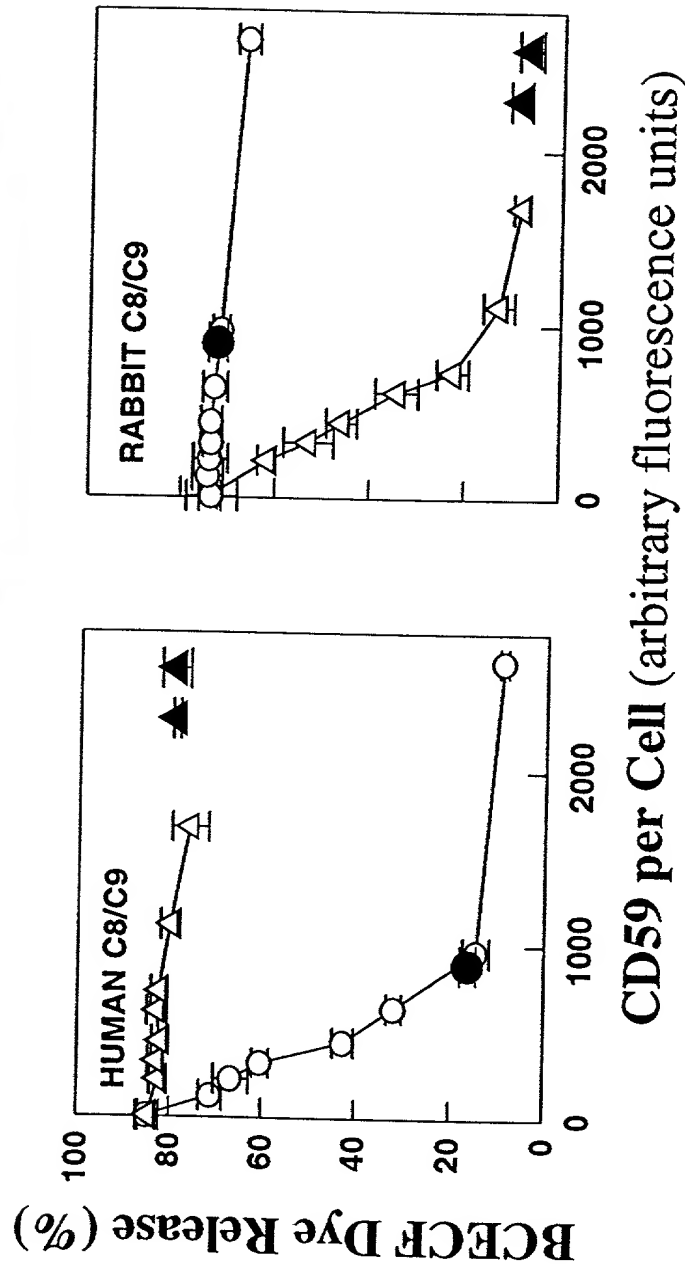
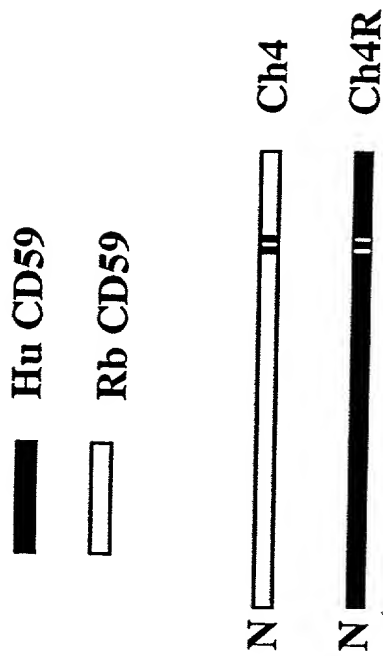
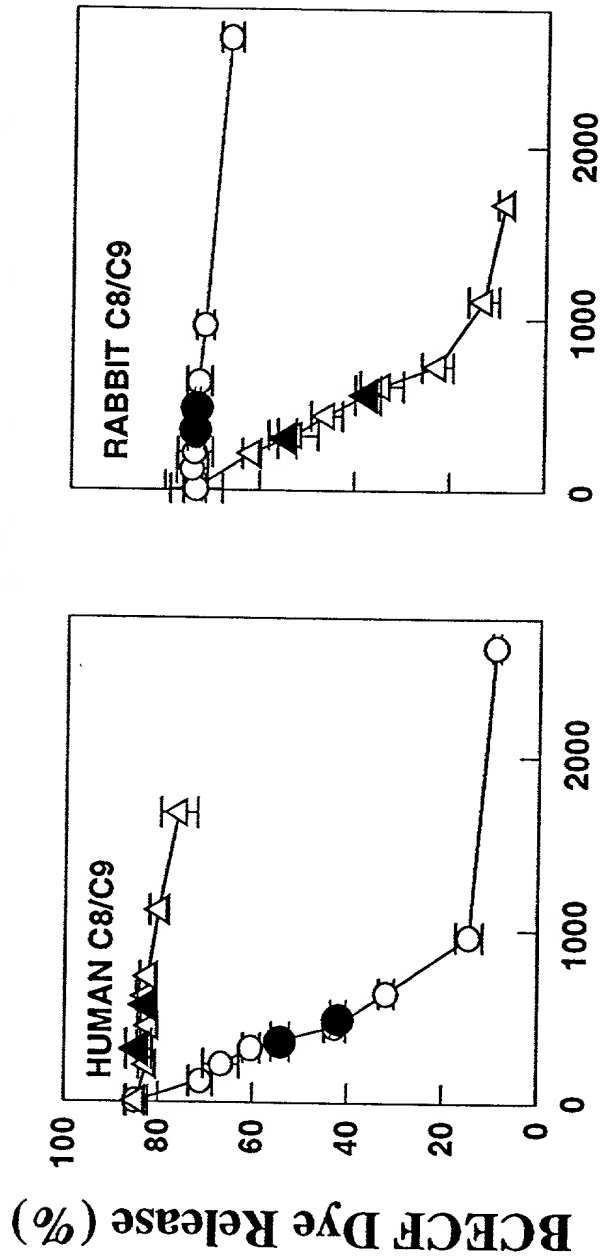
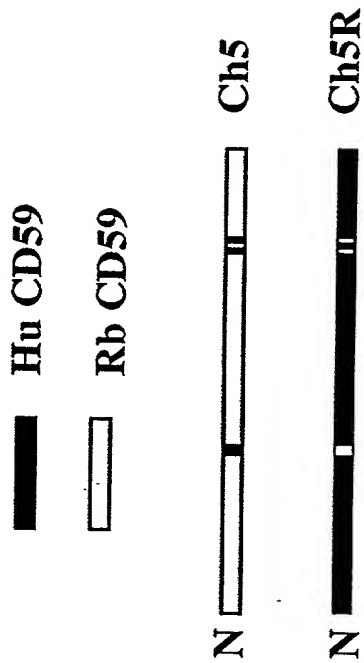


Figure 5C

Figure 6A



CD59 per Cell (arbitrary fluorescence units)

Figure 6B

Figure 6C

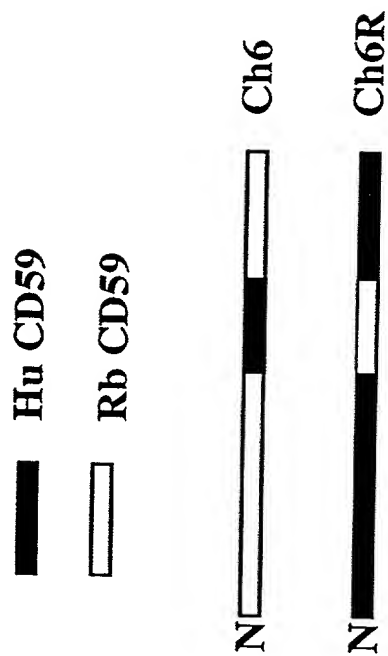


Figure 7A

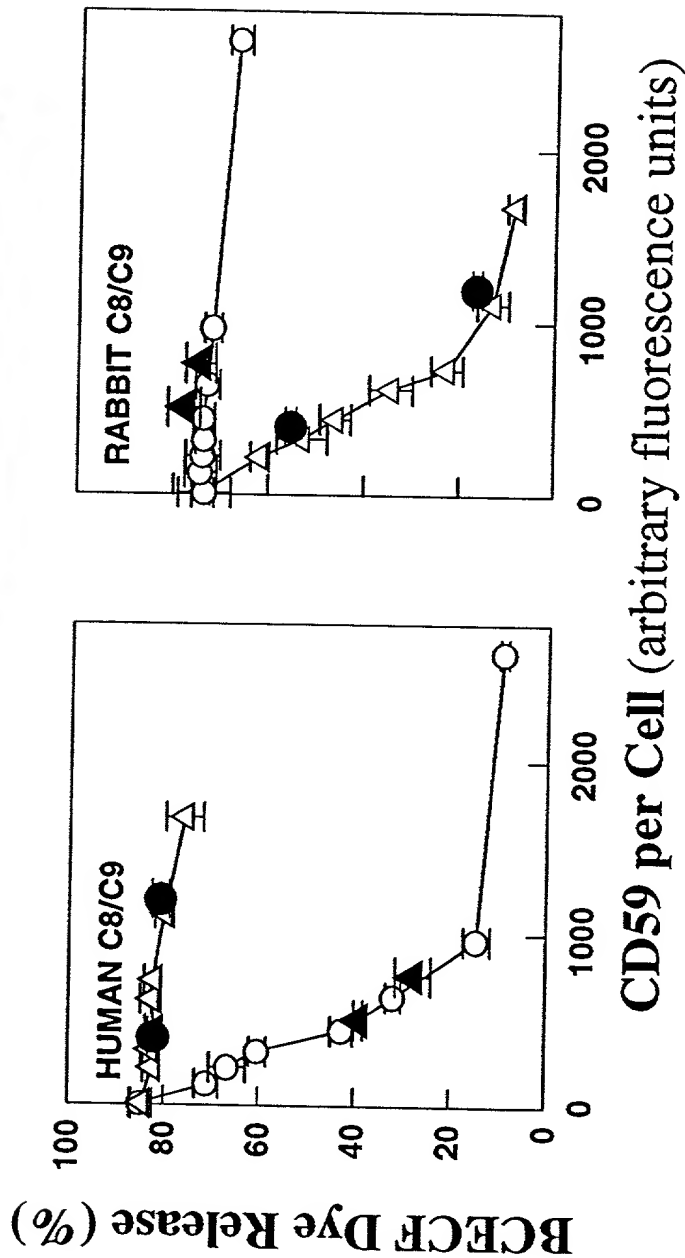


Figure 7B

Figure 7C

	1	5	10	15	20	25	30	35	*							
Human	L Q	C Y N	C P N	P T	A D	C K	A V N	C S	D S	F D	A C L	I T K	A G	L Q	V Y	N K
Baboon	L Q	C Y N	C P N	P T	T N	C K	A I N	C S	G S	F D	T C L	I A R	A G	L Q	V Y	N Q
Afr grn mnky	L Q	C Y N	C P N	P T	T D	C K	A I N	C S	G S	F D	T C L	I A R	A G	L Q	V Y	N Q
Owl monkey	L Q	C Y S	C P Y	P T	T Q	C T M	T T N	C T S	N S	L D	S C L	I A K	A G	S R	V Y	V R
Marmoset	L Q	C Y S	C P Y	S T	A R	C T	T T N	C T S	N S	L D	S C L	I A K	A G	L R	V Y	V R
HVS-15	L Q	C Y N	C S H	S T	N Q	C K	S T S	C T S	N S	L D	S C L	I A K	A G	S G	V Y	N K
Pig	L Q	C Y N	C I N	P A	G S	C T X	X M N	C S Y	N S	Q D	A C I	F V X	A V	P P	K T	
Sheep	L Q	C Y S	C I N	Q Y	D	C T S	V I N	C T Y	N S	Q D	A C L	Y				
Rabbit	L M	C Y H	C L R	S	P N	C S	V T N	C T P	N S	H D	A C L	T A V	S G	P R	V Y	R Q
Rat	L R	C Y N	C L D	P Y	S S	C K	N S T	C S P	N S	L D	A C L	V A V	S G	K Q	V Y	Q Q
Mouse	L T	C Y H	C F Q	P Y	V S S	C N M	N S T	C S P	D S	Q D	S C L	Y A V	A G	M Q	V Y	Q R

	40	45	50	55	60	65	70	71	75
Human	CWK	FEN	VTR	LRN	LTN	CKD	LCN	FN	EN
Baboon	CWK	FAN	IST	LKN	LON	CKD	LCN	FN	EN
Afr grn mnky	CWK	FAN	IST	LKN	LON	CKD	LCN	FN	EN
Owl monkey	CWK	FED	VSN	LKN	LKN	CKD	LCN	FN	EN
Marmoset	CWK	FED	QSN	LKN	LKN	CKD	LCN	FN	EN
HVS-15	CWK	FED	QSN	LKN	LKN	CKD	LCN	FN	EN
Rabbit	CWR	FED	ISN	LEN	LKN	CKD	LCN	VN	KGI
Rat	CWR	FSD	ISN	LEN	LKN	CKD	LCN	GN	EDDG
Mouse	CWK	QSD	IMD	LEN	LKN	CKD	LCN	KSF	EDKPN

Figure 8

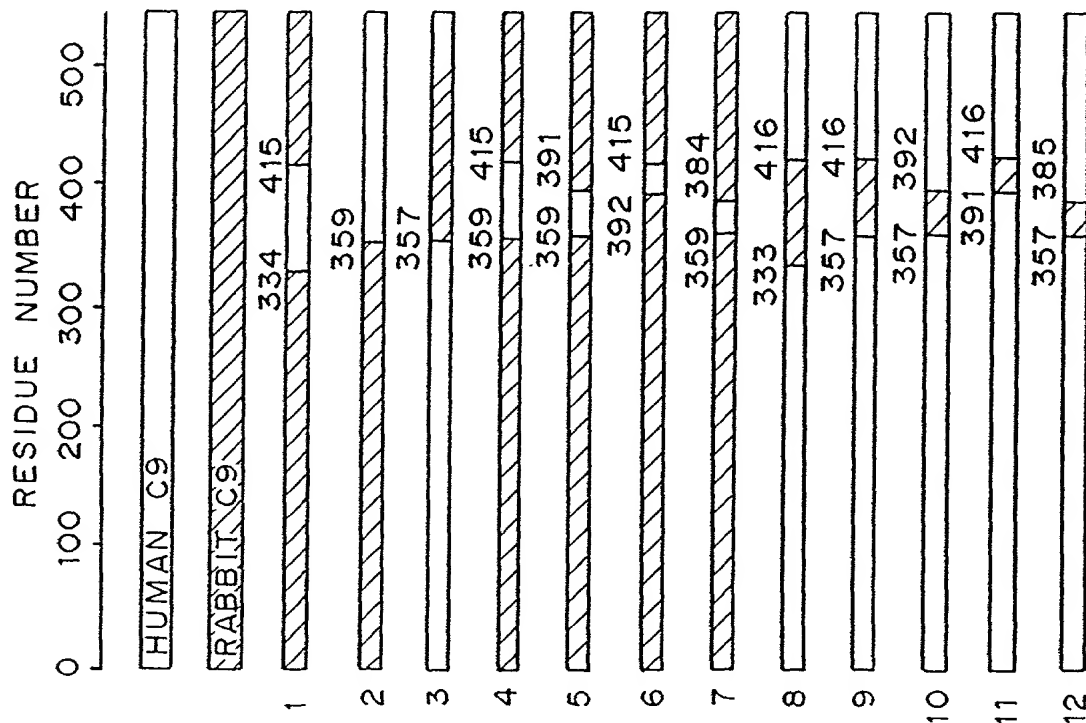


FIGURE 9A

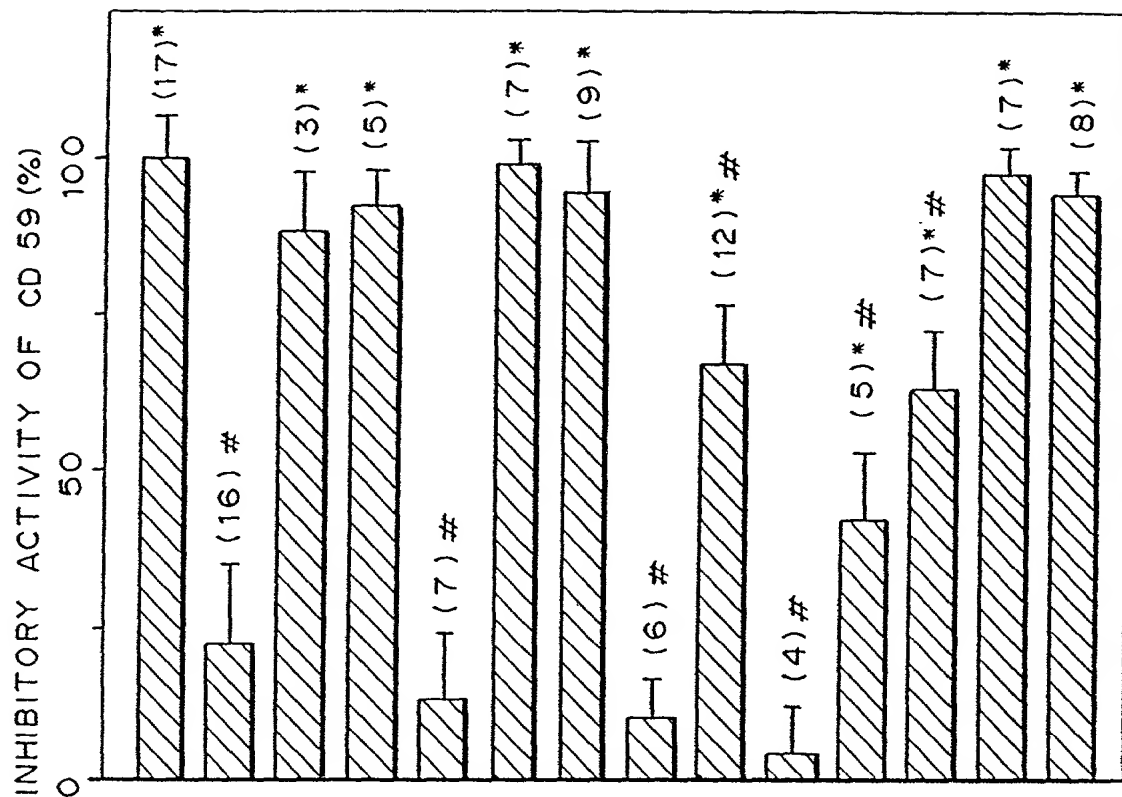


FIGURE 9B

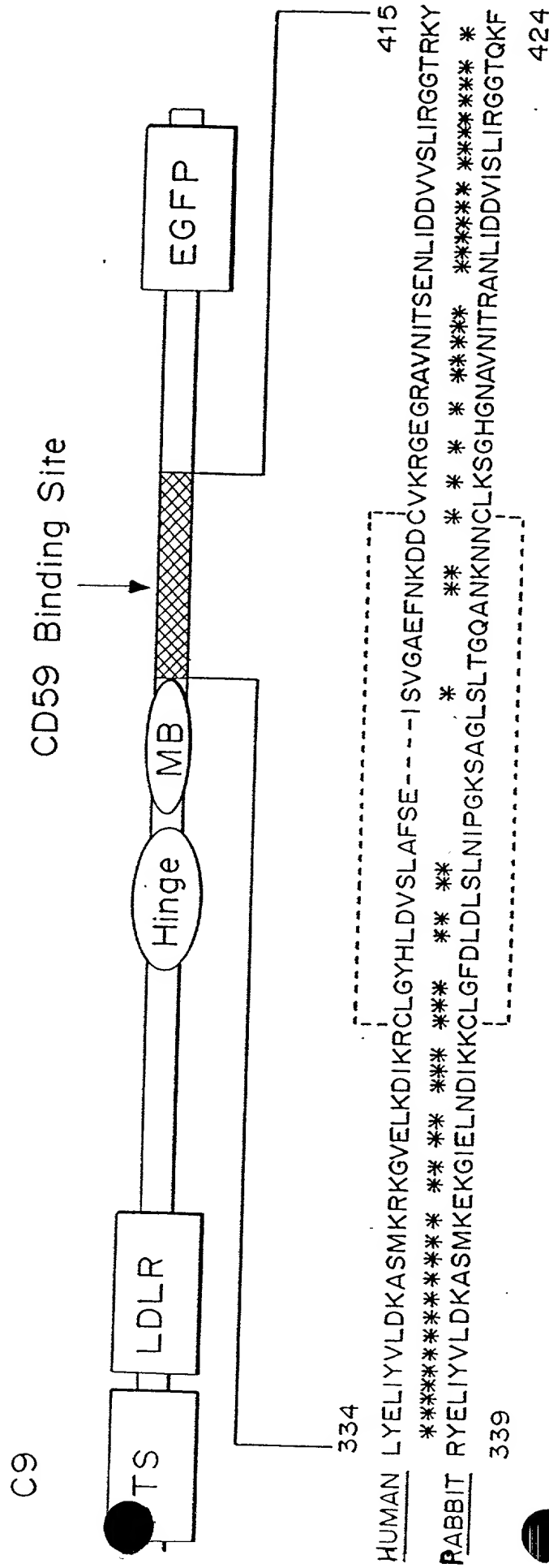


FIGURE 10

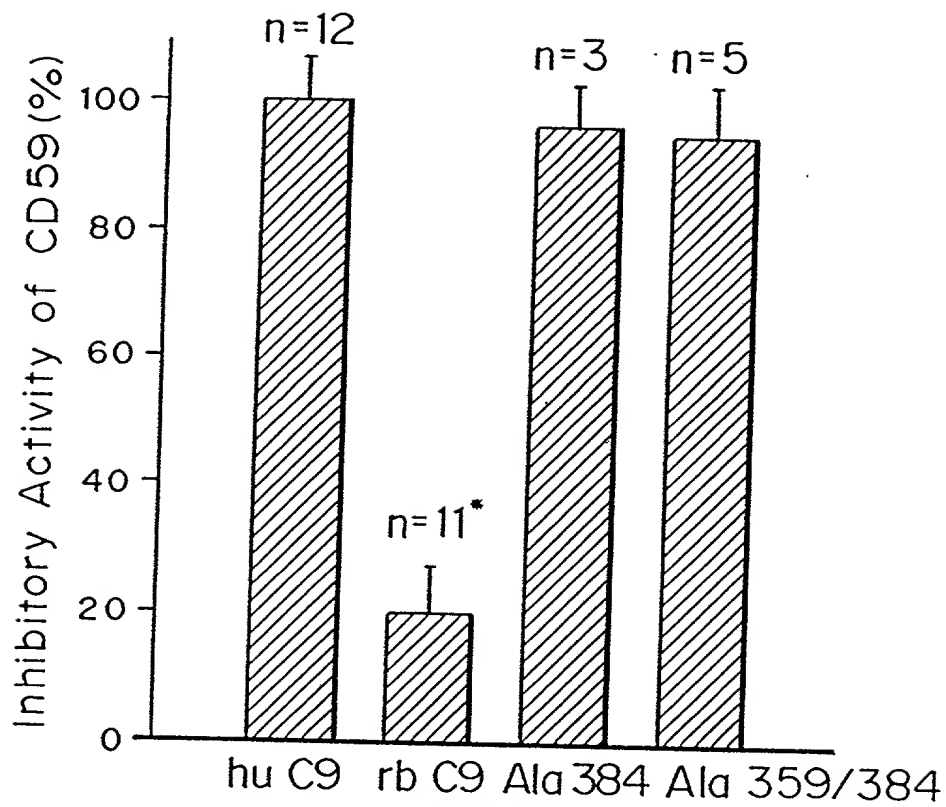


FIGURE 11

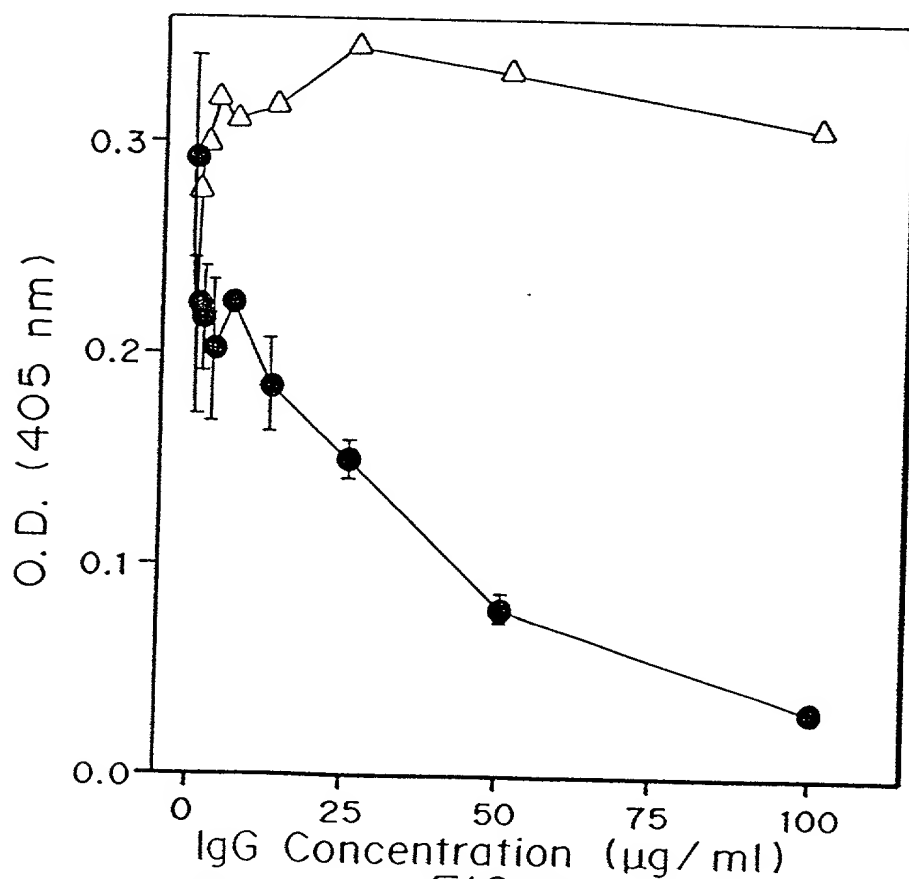


FIGURE 12

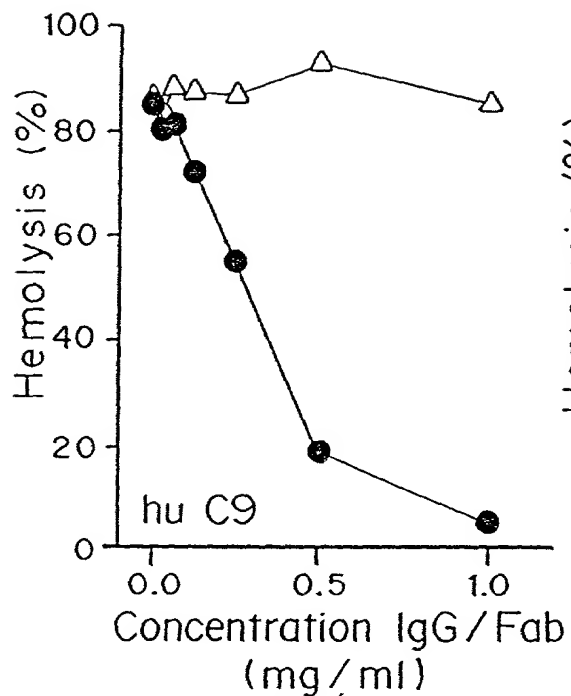


FIGURE 13 A

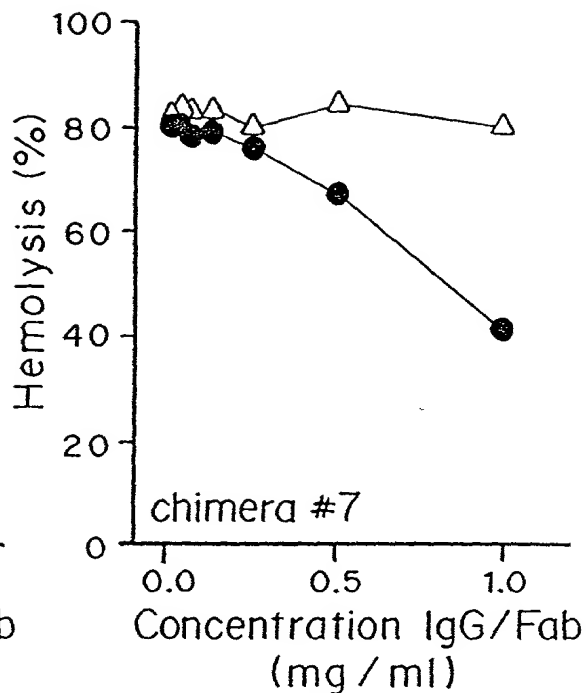


FIGURE 13 B

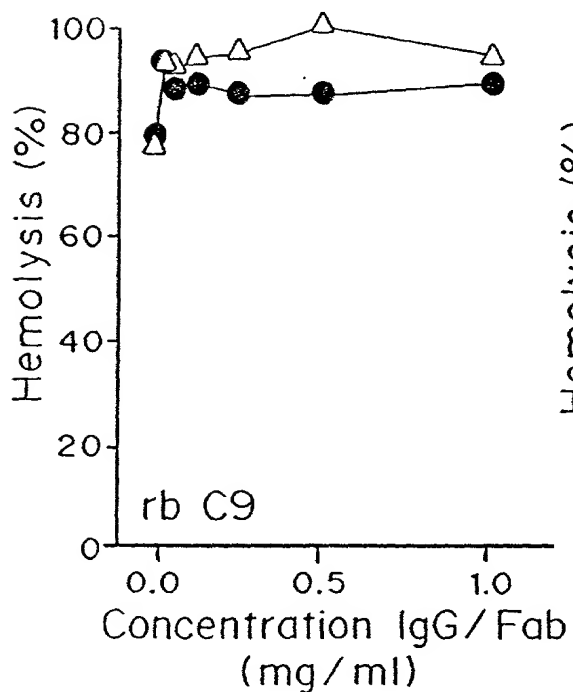


FIGURE 13 C

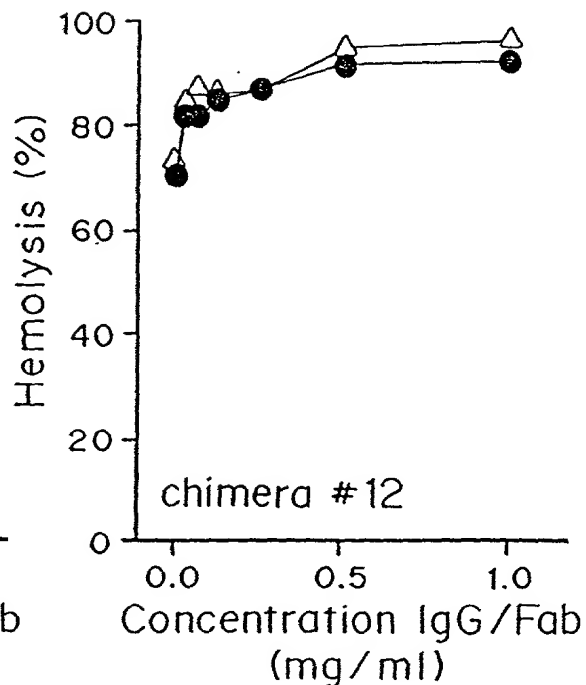


FIGURE 13 D

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below), or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

COMPOSITIONS AND METHODS TO INHIBIT THE FORMATION OF THE C5b-9 COMPLEX OF COMPLEMENT

the specification of which (check one)

_____ is attached hereto

 X was filed on February 9, 1998
as application Serial No. _____

_____ and was amended on: _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)	Priority Claimed
_____ (Number)	_____ (Country)
_____ (Number)	_____ (Country)
_____ (Number)	_____ (Country)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date
_____ (Number)	_____ (Day/Month/Year Filed)
_____ (Number)	_____ (Day/Month/Year Filed)
_____ (Number)	_____ (Day/Month/Year Filed)

Title: "COMPOSITIONS AND METHODS TO INHIBIT THE
FORMATION OF THE C5b-9 COMPLEX OF COMPLEMENT"

By: Peter J. Sims
Filed: February 9, 1998
DECLARATION

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of the application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)

(Filing Date)

Status
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

Status
(patented, pending, abandoned)

As named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Patrea L. Pabst	31,284
Suzanne F. Seavello	37,933
David S. Bradin	37,783
Collen A. Beard	38,824
Robert A. Hodges	41,074
Kevin W. King	P-42,737

Send Correspondence to:

Patrea L. Pabst, Esq.
ARNALL GOLDEN & GREGORY LLP
2800 One Atlantic Center
1201 W. Peachtree Street
Atlanta, GA 30309-3450

Direct telephone calls to:

Patrea L. Pabst (404) 873-8794

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Peter J. Sims

Inventor's signature _____ Date _____

Residence 6815 West Mendota Court, Mequon, WI 53092

Citizenship United States of America

Post Office Address 6815 West Mendota Court, Mequon, WI 53092